EVALUATION OF THE LEVEL OF COAGULATION FACTORS V AND VIII ON STORING FRESH FROZEN PLASMA AT DIFFERENT TEMPERATURES - A STUDY AT REGIONAL BLOOD BANK AND CEmONC CENTRE

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LIST OF ABBREVIATIONS

FFP	-	Fresh Frozen Plasma
CEmONC	-	Comprehensive Emergency Obstetrics and Newborn Care
F V	-	Factor V
FVIII	-	Factor VIII
AABB	-	American Association of Blood Banking
DGHS	-	Director General of Health Sciences
IU	-	International Units
HLA	-	Human Leukocyte Antigen
rFVIII	-	Recombinant Factor VIII
PT	-	Prothrombin Time
APTT	-	Activated Partial Thromboplastin Time
AT	-	Anti Thrombin
DDAVP	-	D-amino D-Arginine Vasopressin
FDA	-	Food and Drug Administration
ACD	-	Acid Citrate Dextrose
CPD	-	Citrate Phosphate Dextrose
CPDA	-	Citrate Phosphate Dextrose Adenine
PF24	-	Plasma Frozen with in 24 hours
PF24RT24	-	Plasma Frozen within 24 hours After Phlebotomy held at room temperature up to 24 Hours After Phlebotomy
ADAMTS13	-	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
TRALI	-	Transfusion Related Acute Lung Injury
TACO	-	Transfusion Associated Circulatory Overload

INTRODUCTION

Fresh frozen plasma has a wide range of applications in day to day clinical practice.[1] It is used to treat coagulation factor deficiencies, actively bleeding patients on long term anticoagulation and to treat the coagulopathy that occurs with massive bleeding.[2,3] Other uses are as an adjuvant therapy in disseminated intravascular coagulation, decompensated liver disease, cardiopulmonary bypass and massive transfusion.[4]

The freezing and storage of FFP is done over a wide range of temperatures. As per the AABB guidelines, freezing and storage settings vary from less than -18^0C up to -65^0C.[5] The guidelines have further stated that attaining a lower temperature for storage may retain the stability of the coagulation factors over a longer duration than standard temperature.[6]

In Tamilnadu, blood bank units have different plasma freezers for storage purposes. Commonly used are -30^0C and -70^0C plasma freezers for FFP preparation and storage. Tertiary care units in the state are equipped with -70^0C freezers whereas majority of the peripheral centers still utilize the -20^0C to -30^0C freezers. This variability is a point of concern with respect to the stability of the coagulation factors.

Transfusion guidelines also add that FFP should be used within a short time after thawing at 30°C to 37°C (6 hrs if stored at room temperature, 24 hrs if stored at 1°C to 6°C).[7]

Once thawed, FFP may not always be transfused immediately and is kept for a period of time at room temperature, many units of such may go unutilized.[8]

The short storage time of the thawed plasma is a definite hindrance in its utilisation. This is further challenged when the expected demands in situations needing FFP transfusions do not comply with the actual supply.

For emergency situations like massive haemorrhage and DIC the immediate availability of FFP may get delayed because of the time required for the thawing procedure. FFP thawed and kept in large amounts in anticipation of an emergency may be wasted if the event does not happen.

If the level of coagulation factors remains in the therapeutic range after a significant duration of storage of thawed plasma, it can be safely used for patients. This will reduce plasma wastage and provide adequate units during emergency situations.

Therefore we have designed this study to compare the stability of coagulation factors in plasma, frozen and stored at -30°C or -70°C for a period of 3 months.

AIM AND OBJECTIVE

AIM

To evaluate the coagulation factors V and VIII levels in thawed plasma, previously frozen and stored at -30°C and at -70°C for 3 months

OBJECTIVE

To analyse the coagulation factors V and VIII levels and in fresh frozen plasma stored at -30°C and at -70°C for a duration of 3 months.

To evaluate whether the thawed plasma stored for a considerable time at 2°C to 6°C is appropriate for therapeutic use.

REVIEW OF LITERATURE

The first apparent plasma transfusion in humans was done in the year 1918 by Frank Hartman to treat Spanish flu.[9] In the third decade of the 20th century, fresh plasma was being routinely used in hospitals to treat blood loss. Later, frozen and dried forms of plasma were found to be effective and easy to store. Fresh frozen plasma was found to be better than dried plasma due to the depletion of prothrombin in the latter.[10]

During the World War II, dried plasma was used to treat trauma victims.[11] After the war, the spectrum of FFP usage broaden to conditions like sepsis (as an antitoxin), burns, nutritional deficiencies, nephrotic syndrome, sickle cell anaemia and childhood acute lymphoblastic leukemia but with varied outcomes.

The first randomized controlled trial on FFP was done in the year 1964 in patients undergoing cardiac surgeries using cardiopulmonary bypass.[12] This was followed by a series of studies on the utility of FFP in a variety of clinical conditions, of which TTP and priming cardiopulmonary bypass pumps are the only proven indications where FFP is found to be beneficial.[13,14,15] It was also found that abnormal coagulation tests in a non-bleeding subject is the most common and inappropriate indication for FFP transfusion.[16,17]

After collection, plasma can be processed as components for transfusion (FFP or PF24) or as source material for further manufacture (recovered plasma or source plasma) into injectable products (plasma derivatives) or non injectable products(reagents).

Various studies have been done before to estimate the probable storage time of thawed plasma at different days of storage and at different temperatures.

FRESH FROZEN PLASMA (FFP)

FFP is plasma obtained from a single donor either by normal donation or by plasmapheresis and rapidly frozen within 6-8 hours of being collected.[18] It contains all coagulation factors and great care must be taken during collection of blood, freezing and thawing to preserve their activity.[2]

Collection of blood:[19,20]

1. Blood should be collected by a clean, single venipuncture.
2. Flow of blood should be rapid and constant.
3. Total time taken to collect 450 ml of blood should not be more than 8 minutes.

Procedure

1. Collect appropriate volume of blood in 350-450 ml CPDA quadruple bags systems

2. Store at 4°C or in air -conditioned room till processed but not for more than 6-8 hours.

3. Place bags in the bucket of the refrigerated centrifuge, balance them accurately and centrifuge at heavy spin (5000 x g for 5 minutes) at 4°C.

4. Express approximately four fifth of the plasma into a satellite bag.

5. Double seal the tube between primary bag and the satellite bag having plasma with metal clips or dielectrical sealer. Separate the satellite bag having plasma.

6. Label the plasma bag and is rapidly frozen. This should be done as soon as possible after collection, in any case within 6-8 hours. The complete freezing process should be as short as possible and preferably should not take more than one hour.

   Rapid freezing can be achieved by spreading the plasma in a thin layer (bags laid flat and not vertical) in freezer at - 70°C or placing the bags protected by a plastic over wrap at - 70°C

7. Most labile coagulation factors are preserved for one year if FFP is kept at -30°C or below. If FFP is not used within one year, it is redesignated as a Single Donor Plasma which can be kept further for 4 years at -30° C or below.

8. Frozen plasma may be thawed by the following method:[21,22,23,24] Place the plasma bag in a plastic over wrap and put in a 37°C circulating water bath (the entry ports of the bag should remain above the water). The FFP should be administered as soon as possible after thawing, and in any event within 12 hours if kept at 2°C to 6°C.

Plasma derivatives

Cryoprecipitate and cryoprecipitate-reduced plasma are synthesized from frozen plasma. Many methods exist for pathogen reduction of plasma.[25] Plasma is utilized for the production of specific plasma protein products through fractionation processes.

FFP can be synthesized from a single unit whole blood collection or by apheresis. FFP must be frozen by 8 hours of collection or earlier in 6 hours if anticoagulated with ACD or as per manufacturer's guidelines.[26,27,28,29,30,31,32,33,34]

FFP has a shelf life of 12 months when stored at –18°C or colder. FFP that is stored at or lesser than -65°C may be stored up to 7 years[35] but such storage requires FDA approval.[36]

Plasma composition

Plasma is the cell-free part of blood composed of water, proteins, electrolytes, lipids, and carbohydrates. It is the transportation medium through which nutrients, hormones, waste products, and drugs are transported through the body. In vivo, the fluidity of plasma is maintained through complex interactions between its procoagulant and anticoagulant proteins and between these proteins, circulating blood cells, and the endothelium.[37]

Characteristics of Plasma - Resuscitation fluid (balanced crystalloid solution)

Osmolarity	-	308 m Osm/l
pH	-	7.4
Na+	-	140 mEq/l
K+	-	4 mEq/l
Cl-	-	100 mEq/l
Mg2+	-	0 mEq/l
Dextrose	-	0-4 g/l
Buffer	-	Protein & Bicarbonate

Factors influencing individual plasma composition[38]

1. Age and gender

- The physiologic aging process is accompanied by an increase in plasma levels of fibrinogen; factors V, VII, VIII, IX, XI, and XIII; protein C; and protein S in both genders.

- A significant decrease of prothrombin activity levels with increasing age has been found in men but not in women.

- Young women have significantly lower AT plasma levels than males of similar age. Because of a marked increase after menopause, AT levels in older women exceed levels in male counterparts.

- Gradually declining AT plasma levels have been observed in males older than 45years.

- Pregnancy is associated with marked increases in levels of fibrinogen, prothrombin, factors VII and VIII, vWF, and factor X, whereas factor V, factor IX, AT, and protein C levels are largely unchanged.

2. ABO blood group

Individuals with blood groups O, A_2O, and A_2A_2 have on average about 20 to 25% lower factor VIII and vWF plasma levels when compared with other ABO blood group constellations.[39,40]

3. Acute phase reaction

An acute phase response caused by any trigger of inflammation results in an increase in plasma levels of alpha-1-antitrypsin, C1 inhibitor, fibrinogen, prothrombin, factor VIII, and vWF and a decrease of albumin levels.

4. Smoking

Smoking causes a low-grade systemic inflammatory response and concomitant increases in plasma fibrinogen.

5. Physical exercise and mental stress

Both acute severe physical exercise and acute mental stress cause increases in fibrinogen, factor VII, factor VIII, and vWF levels. In contrast, prolonged severe physical exercise results in a decrease in factor VII levels, whereas fibrinogen levels continue to be increased during observation.

6. Hormones

- plasma from female donors on estrogen based contraceptives or hormone replacement therapy can appear green due to an estrogen related elevation of ceruloplasmin levels

- Danazol, a weak androgen, improves the synthesis of C1 inhibitor, factor VIII, AT, and protein C.

- Combined oral contraceptives induce increases in plasma fibrinogen; prothrombin; factors VII, VIII, IX, X, and XI; alpha-1-antitrypsin; and protein C, whereas AT and protein S levels decrease.

- Progestogen-only preparations lead to a decrease in factor VIII and an increase in protein S.

- Desmopressin(DDAVP)

    (i) DDAVP has been used successfully to increase factor VIII and vWF levels in plasma donors.

    (ii) The yields of these plasma proteins were markedly improved when cryoprecipitate or factor VIII concentrates were produced from plasma collected after pretreatment of donors with DDAVP.

The influence of storage and freezing on plasma composition[41,42,43]

- Prolonged storage reduces factor VIII levels regardless of hold at 4°C or at room temperature.

- Storage at room temperature however, affect protein S activity, which is substantially less when compared to hold at 4°C.

- Slow freezing at -20°C reduces factor VIII and protein S activities markedly in comparison to rapid freezing to a core temperature of below- 30°C within 1 hour.[36]

- Factor VIII levels decline by 24% even when fresh plasma samples are frozen in liquid nitrogen, stored at-70°C, and thawed at 37°C for 10minutes.

- Plasma frozen rapidly either with the help of blast freezer or using dry ice, dry ice with ethanol and dry ice with antifreeze. Plasma should be thawed at + 30^0 C to +37^0 C in a water bath or by any FDA-cleared devices.

  If we use a water bath for thawing, the plasma component should be placed in a plastic overwrap to prevent contamination. Large number of FFP units collected by apheresis method may require more time for thawing .

- Once thawed, these fresh frozen plasma unit has a shelf life of 24 hours at 1^0C to 6^0 C.

- Thawed plasma kept more than 24 hours must be relabeled as `Thawed Plasma', which can be stored for an additional 96 hours /4 days at 1°C to 6°C

- As per the Council of Europe ‘plasma, fresh frozen’ is defined as plasma prepared from WB or an apheresis plasma.[20] The plasma freezing must be

initiated within 6 hours of collection. If the whole blood is held at 1°C to 6°C it should be frozen within 18 hours. If whole blood or apheresis plasma is rapidly conditioned to 20°C to 22°C following collection, it should be frozen with in 24 hours .Freezing of this plasma units to less than –30°C must be completed within 1 hour.[44] Thus the FFP has an expiry time of 3 years or 36 months if it held at less than –25°C and it may have short expiry of 3 months time if it kept at –18°C to –25°C.

Plasma collection from blood donors

To avoid immunological complications, plasma has to be collected from males, females – never pregnant before and parous female donors whose HLA antibodies test report- negative.

QUALITY OF FFP (FRESH FROZEN PLASMA)

FFP is plasma obtained from a single donor either by normal donation or by plasmapheresis and rapidly frozen within 6-8 hours of being collected. It contains all coagulation factors and great care must be taken during collection of blood, freezing and thawing to preserve their activity.

FFP- Quality as per DGHS[19]

Contents of 1 unit of FFP prepared from 450 ml of whole blood

Plasma	-	175-230 ml
All coagulation factors	-	1 IU/ml of each factor
Fibrinogen	-	200- 400 mg

Dosage of FFP

- about 10ml/kg of body weight. Post transfusion assessment of levels of APTT, PT and Fibrinogen is done for monitoring the effect of FFP.

Storage temperature of plasma

Due to the significant decrease in factor VIII activity between 8 and 24 hours of refrigerated storage, FFP collected in CPD, CP2D, and CPDA-1 is required to be frozen within 8 hours of collection.[45]

FFP collected in ACD is required to be frozen within 6 hours of phlebotomy. If maintained at −18°C or colder, FFP is approved for storage for up to 1 year. If cleared by the FDA, facilities may store FFP for up to 7 years at −65°C or colder. For plasma processed and frozen within 8 to 24 hours of collection, the component is designated as PF24.[46]

PF24 is approved for storage at −18°C for up to 1 year after collection. During transport, FFP and PF24 are maintained in the frozen state by packaging with dry ice in an insulated container.

For transfusion, FFP and PF24 are thawed between 30°C to 37°C in a water bath for approximately 30 minutes or rapidly thawed in an FDA-approved microwave device for approximately 6 minutes.

Different types of plasma

1. Plasma Frozen within 24 Hours After Phlebotomy (PF24)

The plasma that is frozen within 24 hours of collection is defined as, Plasma Frozen within 24 Hours after Phlebotomy (PF24). PF24 has a shelf life of 24 hours

after thawing and stored at 1^0 - 6^0 C. After thawing the PF24,it can be kept for more than 24 hour and has to be relabeled as Thawed Plasma.It can be stored for 4 more days at 1^0 to 6^0C.

2. Plasma Frozen within 24 hours After Phlebotomy held at Room temperature up to 24 Hours After Phlebotomy(PF24RT24) :

The Plasma that is frozen within 24 Hours after Phlebotomy and Held at Room Temperature up to 24 Hours After Phlebotomy is called-PF24RT24 An apheresis plasma unit that is held for up to 24 hours after collection at room temperature and then stored at less -18°C. Once after thawing, the PF24 and PF24RT24 have a shelf life of 24 hours at 1°C to 6°C.

3. Thawed Plasma

Thawed Plasma is defined by FDA as FFP, PF24 or PF24RT24 that has been thawed and held at 1to 6 C for more than 24 hours.[47,48] Thawed Plasma may be held at 1°C to 6°C for 5 days after thawing. In this thawed plasma, fibrinogen, factor 2 level are reduced .Further, the labile factors like, Factor V and Factor VIII are reduced to<60% and <40% from their initial level.

ADAMTS13 levels are maintained in thawed Plasma stored at 1°C to 6°C for 5 days.[49]

4. Quarantine Plasma

This was introduced to prevent transfusion transmitted viral infections in the recipients. According to the Council of Europe, quarantine fresh frozen plasma can be released only after the donor TTI testing result has repeatedly negative for minimum the following viruses –HBV, HCV viruses, HIV-1, and HIV-2 & it is beyond a minimum quarantine period that is greater than the diagnostic window

period of that viral infection, around 6 months. Recent days nucleic acid test - TTI screening for viral infections have reduced the window period for quarantine plasma.

5. Source plasma

It is a licensed product collected by plasmapheresis for the purpose of fractionation into injectable or non-injectable plasma products. Donors of source plasma are not required to be tested for human T-lymphocytic virus (HTLV) 1 and 2 as well as to have a negative result for antibody to hepatitis B core antigen (HBcAg). If intended for injectable products, source plasma is approved for storage at -20°C or colder for up to 10 years.

7. Liquid Plasma

It is separated from whole blood at any time during storage and stored at 1°C to 6°C for up to 5 days after the expiration date of whole blood.

8. Recovered Plasma

Recovered plasma is used for plasma fractionation to obtain plasma derivatives like albumin and immunoglobulins. This recovered plasma is collected by the manufacturers from blood storage centers as liquid plasma and has to be shipped as per the manufacturers specified storage conditions, because there are no defined storage temperatures and expiry time for recovered plasma. This is an unlicensed product.

9. Cryo reduced Plasma

This is a byproduct of cryoprecipitate preparation .Plasma cryoprecipitate reduced must be refrozen within 24 hours at less than –18°C. It has the storage

temperatures of -18°C or below and expiry time of one year which is similar to that of fresh frozen plasma The Cryo reduced plasma has a fibrinogen level of around 200 mg/dL and normal factor V, factor I, factor VII, factor X, antiplasmin, antithrombin, protein C, and protein S. The factor VIII, von Willebrand factor antigen, fibrinogen, and Factor XIII are decreased.[50,51]

8. Pathogen Reduced Plasma

In Plasma pathogens are inactivated by certain methods to obtain these products. Following pathogen reduction techniques are used in European countries: psoralen –methylene blue (amotosalen), Riboflavin[25] and solvent/detergent treatments. Methylene-blue-treated plasma contains approximately 15% to 20% less Factor VIII and fibrinogen than untreated plasma.

Plasma can be treated with the Mirasol system by adding 35 mL of riboflavin and then by illumination for 6 to 10 minutes. Immediately after illumination, the plasma can be released or frozen below –30°C for 2 years. Coagulation factors are well preserved in plasma treated with the Mirasol system.

10. SD plasma[52]

Solvent/detergent-treated plasm is prepared from plasma pool from >1000-25000 donors. This pooled plasma subjected to pathogen reduction with 1% tri-n-butyl phosphate & 1% Triton X-100. This inactivates lipid-enveloped viruses in the SD plasma. Each SD plasma unit contains 200 mL of plasma. Then frozen and stored at –18°C for 12 months. Most coagulation factors are reduced by 10% except Factor VIII (20% reduced) and 50% reduction seen in protein S compared to non-treated fresh frozen plasma.[53]

SD plasma units labeled with the ABO blood group and should be used within 24 hours after thawing.[54]

Cryoprecipitate

Cryoprecipitated antihemophilic factor or `cryoprecipitate' is prepared from Fresh Frozen Plasma. When FFP is thawed to 1°C to 6°C cold insoluble clotting factors are retained and collected by centrifugation then the supernatant plasma is transferred to a satellite bag. The cryoprecipitate is resuspended in the residual plasma approximately 15 mL and is refrozen. This can be stored for 1year at -18°C or below.[55]

Quality Control of Cryoprecipitate : According to AABB Standards, cryoprecipitate must contain 150 mg of fibrinogen/unit and 80 IU of Factor VIII / unit.

As per European standards cryoprecipitate should have 140 mg of fibrinogen/unit , 70 IU of Factor VIII/unit & 100 IU of vWF/unit. It contains the vWF ristocetin cofactor activity of 170 U/bag, Factor XIII and fibronectin.

ADAMTS13 levels are normal in cryoprecipitate. Anti-A and anti-B antibodies are present, but the total amount of these antibodies in single plasma unit is only 1.15%.

Thawed cryoprecpitate must be used as soon as possible and if kept at room temperature for 6 hours as single units.

Plasma for transfusion: Plasma for transfusion can be collected through centrifugation of whole blood or by plasma apheresis. It can then be stored frozen as whole plasma or used to produce more purified constituents, including concentrates of coagulation factors and fibrin sealant, immunoglobulins (normal or specific, e.g.,

Rh immune globulin), anticoagulants (e.g., antithrombin and protein C), complement related proteins (C1-esterase inhibitor), and albumin.

Fresh frozen plasma (FFP) is human donor plasma frozen within a short specified time period after collection (often eight hours) and then stored at a defined temperature, typically at -30°C.

Main recommendations for the use of FP[56]

Recommendations for the transfusion of FP have remained relatively consistent over the years, including:

(1) Active bleeding or prior to surgery or an invasive procedure in patients with acquired deficiencies of one or more coagulation factors as demonstrated by an increased international normalized ratio,[57] prothrombin time, or activated partial thromboplastin time when no alternative therapies are available or appropriate;

(2) Immediate correction of vitamin K deficiency or reversal of warfarin effect in patient with active bleeding, or prior to surgery or an invasive procedure (in conjunction with use of prothrombin complex concentrates.[58]

(3) Disseminated Intra vascular Coagulation/ consumptive coagulopathy with active bleeding

(4) TTP-Thrombotic Thrombocytopenic Purpura

(5) In patients with a congenital factor deficiency planned for any invasive procedure/ surgical procedure, when no alternative therapies are available or appropriate.

Previous common uses of FP that are now considered inappropriate include:

Volume replacement, Correction of hypoalbuminemia or nutritional support and immunoglobulin replacement.

Side effects of Frozen plasma

1. Transfusion-related acute lung injury (TRALI) & transfusion-related circulatory overload (TACO)

More immediate and serious complications of FP are transfusion-related acute lung injury (TRALI) and transfusion-related circulatory overload (TACO), although there are ongoing issues of reporting and diagnosis of these conditions that make accurate estimation of prevalence difficult.[59]

2. Transmission of infectious agents

Main risk of FFP particularly viruses such as Hepatitis B and Hepatitis C viruses, HIV, Parvo virus.

3. Allergic reactions & anaphylaxis

It may occur after transfusion of FFP, of which the most serious is severe anaphylaxis, which may develop in IgA deficient patient with class specific anti IgA.

FIBRINOGEN

Fibrinogen (Factor 1)is one of the important clotting factors in the blood. During tissue injury or active bleeding fibrinogen in the circulating blood is converted into fibrin by thrombin and there by occlude the blood vessels by fibrin clot formation at the site of injury.

Molecular structure

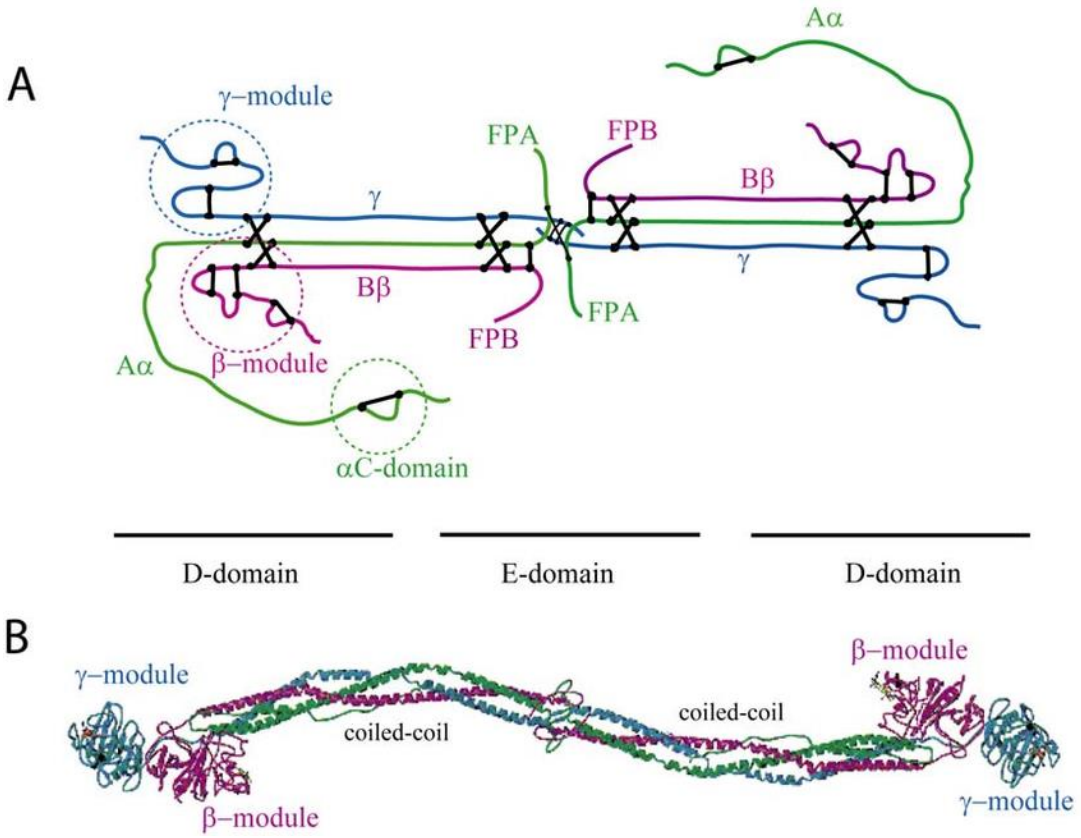


Fig.1: Molecular structure of fibrinogen

Structure of fibrinogen

It has a rod-like shape with dimensions of 9 × 47.5 × 6 nm and has a negative net charge at physiological pH (its isoelectric point is pH 5.8).

Synthesis and Storage: Fibrinogen is made and secreted into the blood primarily by liver hepatocyte cells. Endothelium cells are also reported to make what appears to be small amounts of fibrinogen but this fibrinogen has not been fully characterized; blood platelets and their precursors, bone marrow megakaryocytes, while once thought to make fibrinogen, are now known to take up and store but not make the glycoprotein. The final secreted, hepatocyte-derived

glycoprotein is composed of two trimers with each trimer composed of three different polypeptide chains alpha, beta and gamma chain.

Gene

- The fibrinogen alpha chain (also termed the Aα or α chain) encoded by the c
- The fibrinogen beta chain (also termed the Bβ or β chain) encoded by the FGB gene.
- The fibrinogen gamma chain (also termed the γ chain) encoded by the FGG gene.

Fibrinogen gene Location

All three genes are located on the long or "p" arm of human chromosome 4 (at positions 4q31.3, 4q31.3, and 4q32.1, respectively).

Alternate splicing of the FGA gene produces a minor expanded isoform of Aα termed AαE which replaces Aα in 1–3% of circulating fibrinogen; alternate splicing of FGG produces a minor isoform of γ termed γ' which replaces γ in 8–10% of circulating fibrinogen; FGA is not alternatively spliced. Hence, the final fibrinogen product is composed principally of Aα, Bβ, and γ chains with a small percentage of it containing AαE and/or γ' chains in place of Aα and/or γ chains, respectively. The three genes are transcribed and translated in co-ordination by a mechanism(s) which remains incompletely understood. The coordinated transcription of these three fibrinogen genes is rapidly and greatly increased by systemic conditions such as inflammation and tissue injury. Cytokines produced during these systemic conditions, such as interleukin 6 and interleukin 1β, appear responsible for up-regulating this transcription.

The Aα, Bβ, and γ chains are transcribed and translated coordinately on the endoplasmic reticulum (ER) with their peptide chains being passed into the ER while their signal peptide portions are removed. Inside the ER, the three chains are assembled initially into Aαγ and Bβγ dimers, then to AαBβγ trimers, and finally to (AαBβγ) heximers, i.e. two AαBβγ trimers joined together by numerous disulfide bonds. The heximer is transferred to the Golgi where it is glycosylated, hydroxylated, sulfated, and phosphorylated to form the mature fibrinogen glycoprotein that is secreted into the blood. Mature fibrinogen is arranged as a long flexible protein array of three nodules held together by a very thin thread which is estimated to have a diameter between 8 and 15 Angstrom (Å). The two end nodules (termed D regions or domains) are alike in consisting of Bβ and γ chains while the center slightly smaller nodule (termed the E region or domain) consists of two intertwined Aα alpha chains. Measurements of shadow lengths indicate that nodule diameters are in the range 50 to 70 Å. The length of the dried molecule is 475 ± 25 Å.

Molecular Weight

The fibrinogen molecule circulates as a soluble plasma glycoprotein with a typical molecular weight (depending on its content of Aα verses AαE and γ versus γ' chains) of ~340 kDa.

The normal concentration of fibrinogen in blood plasma is 150–400 mg/dL with levels appreciably below or above this range associated with pathological bleeding and/or thrombosis.

Fibrinogen has a circulating half-life of ~ 4 days.

Function

1. During blood clotting, thrombin attacks the N-terminus of the Aα and Bβ chains in fibrinogen to form individual fibrin strands plus two small polypeptides, fibrinopeptides a and b derived from these respective chains. The individual fibrin strands then polymerize and are cross-linked with other fibrin stands by blood factor XIIIa to form an extensive interconnected fibrin network that is the basis for the formation of a mature fibrin clot. In addition to forming fibrin, fibrinogen also promotes blood clotting by forming bridges between, and activating, blood platelets through binding to their GpIIb/IIIa surface membrane fibrinogen receptor.

2. Fibrin participates in limiting blood clot formation and lysing formed blood clots by at least two important mechanisms. First, it possesses three low affinity binding sites (two in fibrin's E domain; one in its D domain) for thrombin; this binding sequesters thrombin from attacking fibrinogen. Second, fibrin's Aα chain accelerates by at least 100-fold the mount of plasmin activated by tissue plasminogen activator; plasmin breaks-down blood clots. Plasmin's attack on fibrin releases D-dimers (also termed DD dimers). The detection of these dimers in blood is used as a clinical test for fibrinolysis.

FACTOR V

Factor V, also known as proaccelerin or labile factor is a cofactor in blood coagulation. Bleeding disorders occur because of congenital or acquired deficiency of factor V.

Structure

Fig.2: Structure of Factor V

Gene

Factor V gene is located on the first **chromosome (1q23).** It is genomically related to the family of multicopper oxidases, and is homologous to coagulation factor VIII. The gene spans 70 kb, consists of 25 exons, and the resulting protein has a relative molecular mass of approximately 330kDa.

Factor V protein consists of six domains: A1-A2-B-A3-C1-C2 and is similar to the domains of ceruloplasmin. A copper ion is bound in the A1-A3 interface, and A3 interacts with the plasma. The C domains belong to the phospholipid-binding discoidin domain family, and the C2 domain mediate membrane binding. The B domain C-terminus acts as a cofactor for the anticoagulant protein C activation by protein S. Activation of factor V to factor Va is done by cleavage and release of the

B domain, after which the protein no longer assists in activating protein C. The protein is divided to a light and heavy chain, which binds non-covalently to form a complex in a calcium-dependent manner. This complex is the pro-coagulant factor Va.

Synthesis: Factor V synthesis occurs in the liver, principally. The molecule circulates in plasma as a single-chain molecule. Plasma half-life of Factor V is 12 to 36 hours.

Function

Factor V is able to bind to activated platelets and is activated by thrombin. On activation, factor V is spliced in two chains (heavy and light chain with molecular masses of 110000 and 73000, respectively) which are noncovalently bound to each other by calcium. The thereby activated factor V (now called FVa) is a cofactor of the prothrombinase complex: The activated factor X (FXa) enzyme requires calcium and activated factor V to convert prothrombin to thrombin on the cell surface membrane. Factor Va is degraded by activated protein C, one of the principal physiological inhibitors of coagulation. In the presence of thrombomodulin, thrombin acts to decrease clotting by activating Protein C; therefore, the concentration and action of protein C are important determinants in the negative feedback loop through which thrombin limits its own activation.

Factor V deficiency: Factor v deficiency is also known as Owrens disease. Acquired factor V deficiency is a rare condition due to antibody formation against factor V with hemorragic complications.

Structure of Factor VIII

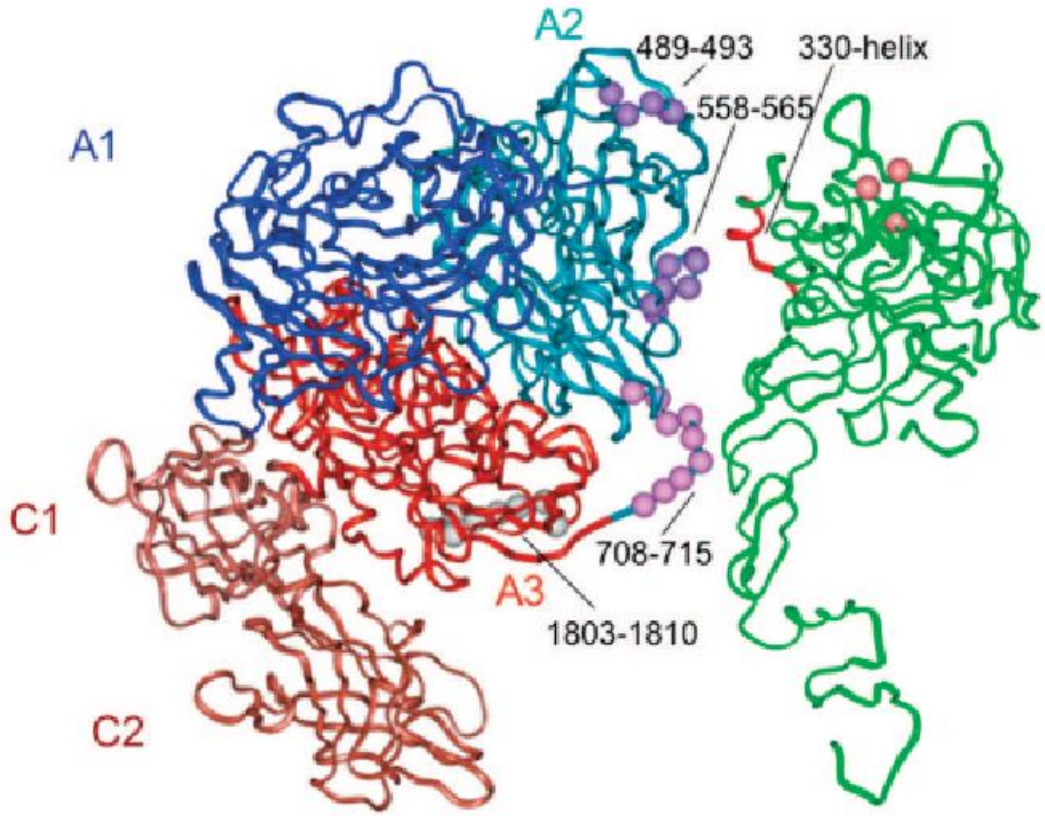


Fig.3: Structure of Factor VIII

Gene

FVIII gene is located in the long arm of X chromosome. It is one of the largest genes known with the molecular weight of 260 kDa.4 It spans over 180 kb. Analysis of the primary structure showed the presence of discrete domain structureA1-a1-A2-a2-B-a3-A3-C1-C2.The A domain display approximately 30% homology to each other, C domains are structurally related to factor V, the B domain is unique ,no significant homology with any other protein. The Factor VIII gene comprises 26 exons which encode polypeptide chain of 2351 amino acids. This includes signal peptide of 19 and a mature protein 2332 amino acids.

Synthesis

Rough endoplasmic reticulum and Golgi apparatus of hepatocytes, are the primary source of FVIII in liver but not in sinusoidal cells and endothelial cells.

SECRETION AND CIRCULATION OF FACTOR VIII

The FVIII is secreted into the circulation, after synthesis in the hepatocytes, in the circulation it forms non covalent bond with vWF. The plasma concentration of FVIII and vWF is 100 to 200 ng /ml and 10µg/ml respectively. vWF binds to the A3 and C2 regions of FVIII through sequence in the D´/D3 region of the mature vWF monomer. In the plasma vWF protects FVIII from proteolysis by activated protein C. Without this interaction the plasma half life of FVIII is reduced and the plasma levels of FVIII are low.

Function: FVIII plays a critical role in the propagation phase of coagulation .Thrombin is the physiological activator of FVIII, which proteolytically cleaves the FVIII at three sites, Arg 372 at the NH2 terminus of the A2 domain, Arg 740 at the NH2 terminus of B domain and Arg 1689 at the NH2 terminus of the A3 domain. These cleavage results in the release of FVIII from vWF and the formation of non covalently associated activated FVIIIa. Activated FVIII forms essential cofactor activity in the intrinsic tenase complex , where FIXa is the serine protease and FX is the substrate. FVIIIa enhances the catalytic reaction about 200,000 fold. Severe deficiency profoundly reduces the rate of generation of FXa.

Two processes are involved in the inactivation of FVIIIa.

1. Spontaneous dissociation of the A2 domain
2. Activated protein C mediated proteolysis at Arg 562 in the FVIIIa heavy chain.

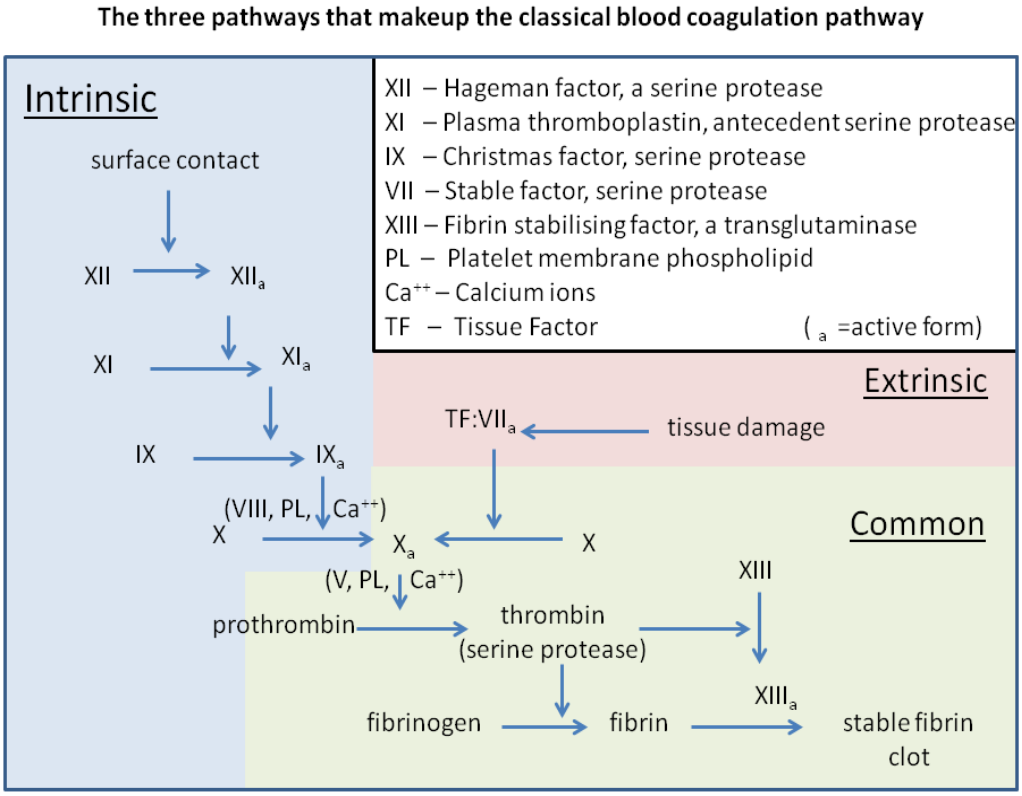


Fig.4: Coagulation Cascade

Role of clotting factors: Coagulation Cascade

The coagulation cascade constitutes the third arm of the hemostatic system.[60] The coagulation cascade is a successive series of amplifying enzymatic reactions. At each step in the process, a proenzyme is proteolyzed to become an active enzyme, which in turn proteolyses the next proenzyme in the series, eventually leading to the activation of thrombin and the formation of fibrin. Thrombin has a key role, as it acts at numerous points in the cascade.

Thrombin proteolyzes fibrinogen into fibrin monomers that polymerize into an insoluble gel; this gel encases platelets and other circulating cells in the definitive secondary hemostatic plug. Fibrin polymers are stabilized by the cross-linking

activity of factor XIIIa, which also is activated by thrombin. Each reaction in the pathway depends on the assembly of a complex composed of an enzyme (an activated coagulation factor), a substrate (a proenzyme form of the next coagulation factor in the series), and a cofactor (a reaction accelerator). These components typically are assembled on a phospholipid surface (provided by endothelial cells or platelets) and are held together by interactions that depend on calcium ions (explaining why blood clotting is prevented by calcium chelators). The sequential cascade of activation can be likened to a 'dance' of complexes, with coagulation factors being passed successively from one partner to the next. Parenthetically, the ability of coagulation factors II, VII, IX, and X to bind to calcium requires that additional γ-carboxyl groups be enzymatically appended to certain glutamic acid residues on these proteins. This reaction requires vitamin K as a cofactor and is antagonized by drugs such as coumadin, which is widely used as an anticoagulant. Blood coagulation traditionally is divided into extrinsic and intrinsic pathways, converging at the activation of factor X. The extrinsic pathway was so designated because it required the addition of an exogenous trigger (originally provided by tissue extracts); the intrinsic pathway only required exposing factor XII (Hageman factor) to a negatively charged surface (even glass suffices). However, this division is largely an artifact of in vitro testing; there are, in fact, several interconnections between the two pathways. The extrinsic pathway is the most physiologically relevant pathway for coagulation occurring after vascular damage; it is activated by tissue factor, a membrane-bound glycoprotein expressed at sites of injury.

Clinical labs assess the function of the two arms of the pathway using two standard assays.[61]

Prothrombin time (PT) screens for the activity of the proteins in the extrinsic pathway (factors VII, X, II, V, and fibrinogen). Because factor VII is the vitamin K–

dependent coagulation factor with the shortest half-life (roughly 7 hours), the PT is used to guide treatment of patients with vitamin K antagonists (e.g., coumadin).

Partial thromboplastin time (PTT) screens for the activity of the proteins in the intrinsic pathway (factors XII, XI, IX, VIII, X, V, II, and fibrinogen). The PTT is performed by adding a negatively charged activator of factor XII (e.g., ground glass) and phospholipids to a patient's citrated plasma, followed by calcium, and recording the time required for clot formation (usually 28 to 35seconds). The PTT is sensitive to the anticoagulant effects of heparin and is therefore used to monitor its efficacy. Once thrombin is formed, it not only catalyzes the final steps in the coagulation cascade, but also exerts a wide variety of effects on the local vasculature and inflammatory milieu; it even actively participates in limiting the extent of the hemostatic process (Fig. 3–10). Most of these thrombin mediated effects occur through protease-activated receptors (PARs), which belong to a family of seven-transmembrane spanning proteins. PARs are present on a variety of cell types, including platelets, endothelium, monocytes, and T lymphocytes. Thrombin activates PARs by clipping their extracellular domains, causing a conformational change that activates associated G proteins. Thus, PAR activation is a catalytic process, explaining the impressive potency of thrombin in eliciting PAR-dependent effects, such as enhancing the adhesive properties of leukocytes. Once activated, the coagulation cascade must be tightly restricted to the site of injury to prevent inappropriate and potentially dangerous clotting elsewhere in the vascular tree. Besides restricting factor activation to sites of exposed phospholipids, clotting also is controlled by three general categories of natural anticoagulants: Antithrombins (e.g., antithrombin III) inhibit the activity of thrombin and other serine proteases, namely factors IXa, Xa, XIa, and XIIa. Antithrombin III is activated by binding to heparin-like molecules on endothelial cells - hence the clinical utility of heparin administration to limit thrombosis.

Protein C and protein S are two vitamin K–dependent proteins that act in a complex to proteolytically inactivate cofactors Va and VIIIa. Protein C activation by thrombomodulin was described earlier; protein S is a cofactor for protein C activity.

Tissue factor pathway inhibitor (TFPI) is a protein secreted by endothelium (and other cell types) that inactivates factor Xa and tissue factor–factor VIIa complexes. Clotting also sets into motion a fibrinolytic cascade that moderates the ultimate size of the clot. Fibrinolysis is largely carried out by plasmin, which breaks down fibrin and interferes with its polymerization. The resulting fibrin split products (FSPs or fibrin degradation products) also can act as weak anticoagulants. Elevated levels of FSPs (most notably fibrin-derived *D-dimers*) can be used for diagnosing abnormal thrombotic states including disseminated intravascular coagulation (DIC), deep venous thrombosis, or pulmonary thromboembolism.

Plasmin is generated by proteolysis of plasminogen, an inactive plasma precursor, either by factor XII or by plasminogen activators. The most important of the plasminogen activators is tissue-type plasminogen activator (t-PA); t-PA is synthesized principally by endothelial cells and is most active when attached to fibrin. The affinity for fibrin largely confines t-PA fibrinolytic activity to sites of recent thrombosis. Urokinase-like plasminogen activator (u- PA) is another plasminogen activator present in plasma and in various tissues; it can activate plasmin in the fluid phase. In addition, plasminogen can be cleaved to its active form by the bacterial product streptokinase, which is used clinically to lyse clots in some forms of thrombotic disease. As with any potent regulatory component, the activity of plasmin is tightly restricted. To prevent excess plasmin from lysing thrombi indiscriminately throughout the body, free plasmin rapidly complexes with circulating α2-antiplasmin and is inactivated.

Endothelial cells further modulate the coagulation– anticoagulation balance by releasing plasminogen activator inhibitors (PAIs); these block fibrinolysis and confer an overall procoagulation effect. PAI production is increased by inflammatory cytokines (in particular interferon-γ) and probably contributes to the intravascular thrombosis that accompanies severe inflammation.

Naghadeh and Roudkenar[32] in 2009 aimed to compare the level of coagulation factors in FFP produced from whole blood and stored at 4 degree celcius for 24 hrs with FFP separated from whole blood within 8 hrs of donation. They observed that the concentration of FVIII in the 24 hr plasma is 82% of the FFP units prepared within 8 hrs of blood collection but is within the therapeutic range. They concluded that plasma produced from whole blood stored at 4 degree celcius for 24 hrs would be an acceptable product for patients requiring FFP.

Naghadeh and colleagues[62] in 2011 collected samples from thawed plasma units stored for 5 days at 1°C to 6°C to assess the levels of factors V, VIII and X and to measure activated partial thromboplastin time and prothrombin time. They observed that the average decreases in the % of coagulation factors from day 1 to day 5 were 20% for V, 11% for X and 25% for factor VIII. Though the changes were statistically significant, the values were within the hemostatic range. They also observed that the increases in the levels of PT and APTT over the period were not clinically pathological and hence concluded that thawed FFP stored for 5 days at 1°C to 6°C can be used in coagulopathy.

Zongkui Wang and colleagues[63] done a study in 2014, were evaluated coagulation related proteins in thawed apheresis FFP units during 5 days of storage at 1-6°C. In that study apheresis fresh frozen plasma units were stored at -70°C, further thawed at 37°C and stored at 1°C to 6°C for 0, 1, 2, 3, 4 and 5 days. Prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time

(TT), fibrinogen level, factor II, V, VII, VIII, IX, X, XI, XII, protein C, protein S, antithrombin III and ADAMTS13 levels were assessed at Days 0-5,

There was no significant differences were observed in Fibrinogen, Protein C, Protein S, Anti thrombin III and ADAMTS13. But Factors II, V, VII, VIII, IX, X, XI and XII declined significantly over time. On storage decrease for FVIII about 40% from day 0was observed. Other factors were maintained around 60% when compared to base line day 0 levels.

Yazer MH and co workers[27] done a study on Coagulation factor levels in FP24 (Plasma frozen within 24 hrs of phlebotomy) for 5 days at 1°C to 6°C. They compared the FFP (Plasma frozen within 6 hrs of phlebotomy) and FP24 for coagulation factors level on 2,4 and 5days of storage at 1°C to 6°C. They found that Factors II, V, VII,VIII,IX ,XI,XII ,ADAMTS-13, Pr C and AT III levels were well maintained in their therapeutic range and Pr S level was reduced on 5^{th} day of storage.

Sheffield et al[64] did an analysis in the year 2012, regarding the coagulation factor activity and di ethyl hexyl phthalate concentration in frozen plasma thawed and stored at 1-6degree for upto 5 days. Sampling was done at 0, 24, 72 and 120 hours after thawing. They observed that factor V and factor VIII levels declined significantly within 24 hours. At day 5, factor VIII had a 41% loss and factor V had 14% loss. Mean di- ethyl hexyl phthalate levels rise at day5.

William Sheffield, Varsha Bhakta, Qi-long Yi and Craig Jenkins[47] in the year 2016, studied the stability of thawed apheresis FFP stored for upto 120 hrs at 1°C to 6°C and published their observation in ‘Journal of Blood Transfusion’. They compared the level of coagulation factors, fibrinogen activities and prothrombin time between thawed refrigerated FFP following apheresis donation (FFPA) and

thawed refrigerated frozen plasma (FP) following whole blood donation at 0, 24 and 120 hours of storage. They conducted that factor activities were significantly higher in FFPA group than FP group after 120 hours of refrigerated storage.

Nilsson and colleagues,[65] way back in 1983 tried to analyse the stability of coagulation factors in whole blood and plasma stored at 4℃ over a period of time. They observed that factor VIII coagulant activity was normal for the first 6 hours of storage of whole blood. The maximum fall (50% of original value) happened in 6 to 24 hours and thereafter no significant fall was seen. In contrast the fall in factor VIII coagulant activity to 50% of its original value occurred at 7 – 14 days of storage in plasma. There was no significant decrease in the values of other coagulation factors. They concluded that storage of whole blood and plasma over 1 – 2 weeks had no effect on the values of coagulation factors with the exception of factor VIII coagulant activity.

Cardigan R and others[34] in 2005 compared the level of coagulation factors between Fresh-frozen plasma(FFP) separated from whole blood leukodepleted (LD) after storage at 4℃ overnight (18-24 hr from donation, Day 1 FFP) and LD within 8 hours of donation (Day 0 FFP). They opined that there is good retention of relevant coagulation factor activity in plasma produced from whole blood stored at 4℃ for 18 to 24 hours and that this would be an acceptable product for most patients requiring FFP.

Cardigan R and Green L[66] have published a review article in 'Vox Sanguinis' in 2015 about the efficacy of coagulation factors, thrombin generation and the levels of DEHP (Diethyl hexyl phthalate) in frozen thawed plasma stored over a period of time. They have suggested that the potential reduction the efficacy of the extended thawed plasma should be balanced with the clinical need for the component.

A Ettinger, MM Miklauz and others[25] in 2011 performed an in-vitro study to evaluate the protein quality of previously frozen FFP, thawed, treated with riboflavin and UV light (Mirasol treatment) and refrozen for a final storage period of up to 2 years at 30℃. They concluded that Riboflavin and UV light-treated FFP maintained both coagulant and anticoagulant in-vitro protein quality after double freeze/thaw storage at 30℃ for up to 2 years and this may offer processing flexibility to blood centers.

Buchta C and his associates[52] investigated the stability of coagulation factor activities and plasma protein levels during 6 days of storage of thawed solvent/detergent (S/D)-treated plasma at +4 degrees C in the year 2004. They observed that except for protein S, the activities of all coagulation factors and inhibitors were at least 0.5 U/ml during storage at 4 degrees C for 6 days. The mean levels, during storage, of factors IX, X, XI and XII, vWF: Ag, fibrinogen and protein C were at least 94%, and of factors II, V and VIII, and AT atleast 78%, of the levels immediately after thawing; the activity of factor VII decreased to 83% and of protein S to 43% of the baseline values. These findings made them conclude that thawed S/D-treated plasma stored at +4°C for up to 6 days still contains sufficient coagulation activities and plasma proteins to be regarded as suitable for transfusion in the established indications.

Alesci S, Borggrefe M and Dempfle CE[35] performed a detailed study on the various coagulation times and fibrinogen levels in plasma frozen and maintained at different temperatures. Prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen using a kinetic fibrinogen assay, PT-derived fibrinogen, and an immunoassay were measured in fresh plasma samples from 16 healthy blood donors. In addition, four sets of aliquots were prepared. They observed that PT and aPTT were influenced to a greater degree by freezing and storage. Fibrinogen levels

were the least affected. In addition they observed that frozen and thawed samples generated slightly higher fibrinogen levels compared to fresh samples. They opined that Prothrombin time and aPTT should be measured in fresh samples.

Dzik WH, Riibner MA and Linehan SK[67] conducted a study to assess the stability of coagulation factors V and VIII:C in previously thawed and refreeze fresh frozen plasma. Fifty-eight units of plasma were studied, with each experimental unit of FFP paired with an identical control unit. Experimental units were frozen, stored at -65°C, thawed, stored at 1°C to 6°C for various periods of time up to 24 hours, and then refrozen, stored at -65°C, rethawed, and stored again in the refrigerator for up to 24 hours. Control units were frozen once at the time the experimental units were first frozen and thawed once at the time of the second thaw of the experimental units. The results of coagulation testing of the twice-frozen plasmas were always within the normal range. There was a slight but statistically valid prolongation of the PT and aPTT and a decrease in the factor V and VIII:C levels for twice-frozen plasma compared with control plasma. The greatest decline occurred in the level of factor VIII:C. The measured deterioration in coagulation of twice-frozen FFP is unlikely to be of clinical importance. They concluded that refreezing FFP might eventually prove useful for rare donor, autologous and massive transfusion programs.

The effects of storage and thawing conditions on coagulation testing was analysed by Gosselin RC, Honeychurch K, Kang HJ and Dwyre DM[68] in the year 2015. They measured the effects of freezing and thawing conditions 3.2% buffered sodium citrate plasma samples that have been stored in vials with either snap or sealed screw tops, frozen in -70°C freezer or dry ice and thawed either capped or uncapped. Their results say that the type of storage vials, freezing and thawing

condition affect coagulation testing, although these differences may not be clinically significant.

Isaacs MS, Scheuermaier KD and others[21] studied the in vitro effects of thawing fresh-frozen plasma at various temperatures and published their findings in 2004. In their study, fifteen adult units of FFP were each divided into 4 satellite units by the South African Blood Transfusion Service before freezing at -25°C. These bags were then defrosted in a waterbath at 22°C, 37°C, 45°C and 60°C, respectively, and removed as soon as they had thawed. Samples were collected for measurement of International Normalized Ratio (INR), prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, and D-dimers. They summarized that INR and PTT values increased at a thawing temperature of 60°C compared with 37°C and fibrinogen levels were markedly decreased in FFP thawed at 60°C compared with that thawed at 37°C and further added that FFP should be thawed at 37°C in a strictly controlled environment.

The effect of time gap between blood collection and freezing of plasma on the stability of clotting factors was studied by Kellner S[29] in 1985. Plasma samples were divided in two groups and flash frozen at -40°C within 6 hours and within 18 hours after phlebotomy. Coagulation factors II, V, VII, VIII, IX and X as well as AT III, APTT and Quick were measured. Their results demonstrated that only minor differences exist in activity of coagulation factors of 6 hours and 18 hours plasma.

Neisser-Svae A and coworkers[46] compared the five-day stability of solvent/detergent-treated thawed plasma with that of fresh-frozen plasma and plasma frozen within 24 hours. The activity of factor (F)V, FVII, FVIII, protein S (PS), and ADAMTS13 was determined in each unit at baseline and every 24 hours thereafter for 5 days. They concluded that over 5 days of refrigerated storage, the changes in the measured coagulation factors in the groups of fresh frozen plasma,

plasma frozen after 24 hours of collection and solvent-detergent treated plasma groups are comparable.

Smak Gregoor et al[50] performed a study using CPD FFP and CPD Cryo supernatant plasma (plasma without cryoprecipitate) which were stored at 4°C for a period of 28 days. Fibrinogen levels remained constant over the period of testing, whereas factor V decreased to 58% and factor VIII fell to 36% of its baseline value. Prothrombin time increased on an average by 2.5 seconds. They concluded that FFP can be stored at 4°C for upto 28 days.

Heil W[69] concluded that analysis of coagulation factors in freshly prepared plasma should be performed within 4 hours of collection for obtaining accurate values.

Kakaiya RM[41] and associates demonstrated that FFP frozen within 18 hours of phlebotomy retained stability of all coagulation factors similar to that frozen within 6 hours of phlebotomy. The exception was factor VIII levels which showed a decrease of 55% from baseline in the 18 hour group.

Lamboo M et al[70] observed that thawed FFP stored at room temperature showed a fall in F VIII to 16% and APTT prolongation of 6% at 6 hours of storage. Similarly, FFP stored at 4°C for 2 weeks showed a decrease in F VIII by 45% and an increase in APTT by 17%. They concluded that thawed FFP stored at 4°C for 2 weeks can help in attained hemostasis on administration in patients with Thrombotic thrombocytopenic purpura (TTP) except in patients with known deficiency of F VIII.

Noordin SS and associates[71] analysed the changes in coagulation factor activities over a period of 5 days in thawed FFP which has been stored at two different initial storage temperatures. The first group had thawed FFP stored at 2°C

to 6°C for 5 days and the second group had FFP stored at 20°C to 24°C for initial 6 hours followed by 2°C to 6°C for 5 days. They observed that coagulation factor activities were reduced in both the groups but were still above 30% and suggested that thawed FFP can be stored for 5 days and can be used in patients to treat factor deficiencies.

MATERIALS AND METHODS

Study Design

Prospective study

Period of Study

0ctober2017 –September2018

Sample Size

40 (calculation done using the formula)

Sample Size Estimation

$$Npairs = \frac{(Z\alpha+Z\beta)^2}{\Delta^2} + \frac{Z\alpha^2}{2}$$

$$\Delta = \frac{Mean_1 - Mean_2}{Standard\ deviation}$$

$$SD = \frac{SD_1 + SD_2}{2}$$

Mean 1 = day 1 mean F V

Mean 2 = day 5 mean F V

SD1 = Standard deviation on day 1

SD2 = S Standard deviation on day 5

Δ = Effect size

α = Level of Significance = 5% = 1.96

β = power = 80% or 90%

Inclusion Criteria

- Blood units collected by proper aseptic methods with in appropriate time (6-8 minutes)
- Plasma components prepared within 6-8 hrs of collection, frozen at different temperatures (-30^{0}C and at -70^{0}C) were taken for study.

Exclusion Criteria

- Low volume collections.
- Lipemic collections.
- Plasma units with RBC contamination.

Procedure

- Donor units collected in Quadruple bags were randomly selected for study purpose.

- As per NACO guidelines 1% of the collection units or maximum of 4 units/month have been taken for our study purpose after ethical clearance obtained from the Institutional Ethics Committee

- The selected whole blood units were subjected to component separation within 6-8 hrs of collection and the separated plasma gets collected in secondary bag.

- Twenty fresh frozen plasma units from healthy donors collected in the Department of Transfusion Medicine, The Tamilnadu Dr.MGR Medical University (Regional Blood Bank), Guindy, Chennai and Twenty fresh frozen plasma units from healthy donors collected in the Department of Transfusion Medicine, Government Kilpauk Medical College and Hospital(

CEmONC-Comprehensive Emergency Obstetric and Newborn Care centre), Kilpauk, Chennai, were randomly selected.

- The selected whole blood units were subjected to component separation within 6 to 8 hours of collection and the separated plasma gets collected in secondary bag.
- Initial baseline values of factor V, VIII and fibrinogen levels in the selected units were measured
- PT and APTT values also were measured in the plasma units
- The plasma units were stored at -30°C and at -70°C in plasma freezer
- After 3 months of storage , the selected FFP units were thawed at +30°C to +37°C FV, FVIII, fibrinogen levels and PT, APTT values were measured in the thawed plasma on day 0,day 1,day 3 and day 5 of storage at 2°C to 6°C.
- Sampling of plasma for measurement of factor levels on every occasions were done by stripping followed by mixing the bag contents
- Clotting factor V, VIII, Fibrinogen levels and PT, APTT values were measured using Coagulometer (Erba ECL 412, TransAsia Bio Medicals).
- Results were statistically analysed using SPSS version 17 software.

Sample Collection for the evaluation of Coagulation Factor Activities in FFP

Initial level of coagulation factor activities in the selected plasma units before kept in the freezer was noted. Then the plasma units were aliquoted in to 2 bags and kept at -30°C and at -70°C. Those units were kept in that particular storage temperatures for up to 3 months duration. After that those FFP units were taken and

thawed at + 37°C in a thawing bath. Coagulation factor activities in the thawed FFP units were tested immediately after thawing and noted as day 0 values (0 hrs value). Further, the same reading were taken after 24 hrs and noted as day 1(24 hrs value). After the 24 hour expiration time, thawed fresh frozen plasma were kept at 2°C to 6°C for 4 more days. Samples were taken at day 3 (72 hrs value) and day 5 (120 hrs value) of storage at 2°C to 6°C for study purpose.

Coagulation Factor Assays

Assays for coagulation factor V, factor VIII and fibrinogen were performed with Erba Kit (Transasia Bio Medicals Ltd, Daman, India) on a ECL 412 semi automated coagulometer (Transasia Bio-Medicals Ltd,India)

The assay for fibrinogen was performed by the clotting method of Clauss[72] using the Erba-Thrombin Reagent kit on a ECL 412 semi automated coagulometer.

The assay for factor V and factor VIII were performed by using the factor V deficient plasma (Erba Bio-Medicals) and factor VIII deficient plasma (Erba Bio-Medicals) kit provided for factor assay, using ECL 412 semi automated coagulometer.

The assay consists of determination of prothrombin time (PT), which measures the clotting time of the fresh frozen plasma and compares it with that of a normal standard was performed using the Erba- kit with the help of an ECL 412 semi automated coagulometer.

The assay consists of the determination of activated partial thromboplastin time (APTT), which was performed using the Erba- kit with the help of an ECL 412 semi automated coagulometer. A standard curve was run with each assay. Calibration material was prepared from a standard plasma(Erba-Std plasma).

Procedures

Fibrinogen Assay Principle

Clauss method: This is a modified TT test in which prediluted plasma (1:10 in Owren buffer) is mixed with a thrombin solution (100 micro L). In these conditions, the time of fibrin polymerization is inversely proportional to the clottable protein content. This method was performed by semi-automatic equipment with electro-optical detection of the clot . The results obtained were statistically analysed.

Method

- 10 µl plasma to be tested was taken in a test cuvette
- The sample was diluted with 90 µl of OV(Owrens Veronal) buffer (1:10 dilution)
- It was mixed well and 100 µl of diluted sample was taken in a test cuvette
- It was kept in the test channel and incubated for 2 minutes
- 50 µl of Thrombin reagent was added

The result in the display was noted- it gave the %activity of fibrinogen in the given sample.

Factor V assay Principle

Quantitative measurement of individual coagulation factors by the one-stage method requires substrate plasma lacking the factor to be measured. A dilution of the test plasma is mixed with the factor deficient plasma and the clot time of the mixture determined. The degree of clot time correction with the patient plasma is compared to the correction with a reference material, allowing the % activity of the patient plasma to be determined.

Method

- 10 µl plasma to be tested was taken in a test cuvette
- The sample was diluted with 90 µl of OV(Owrens Veronal) buffer (1:10 dilution)
- It was mixed well and 40 µl of diluted sample was taken in a test cuvette
- It was kept in the test channel and incubated for 2 minutes in Erba ECL 412 analyzer
- 80 µl of PT reagent was added

The result in the display was noted- it gave the % activity of factor V in the given sample.

Factor VIII assay Principle

Quantitative measurement of individual coagulation factors by the one-stage method requires substrate plasma lacking the factor to be measured. A dilution of the test plasma is mixed with the factor deficient plasma and the clot time of the mixture determined. The degree of clot time correction with the patient plasma is compared to the correction with a reference material, allowing the % activity of the patient plasma to be determined(.ref-Penner JA)

Method

- 10 µl plasma to be tested was taken in a test cuvette
- The sample was diluted with 40 µl of OV(Owrens Veronal) buffer
- It was mixed well and 40 µl of diluted sample was taken in a test cuvette
- 40 µl of factor VIII deficient plasma was added to the diluted sample
- It was incubated it for 2 minutes as programmed in Erba ECL 412 analyzer
- 40 µl of APTT reagent was added

- It was incubated it for 2 as programmed in Erba ECL 412 analyzer
- 40 µl of $CaCl_2$ (0.025M Calcium Chloride) was added
- It was incubated it for 4 minutes as programmed in Erba ECL 412 analyzer

The results in the display were read – it gave the % activity of factor VIII in the test plasma.

Prothrombin time (PT)

Prothrombin Time measures the functioning of factors in the extrinsic pathway (factors VII, X, II, V, and fibrinogen).

Principle

The PT is performed by adding phospholipids and tissue factor to a patient's citrated plasma (sodium citrate chelates calcium and prevents spontaneous clotting), followed by calcium, and the time to fibrin clot formation is recorded

Method

- 50 µl plasma to be tested was taken in a test cuvette
- It was incubated it for 2 minutes as programmed in Erba ECL 412 analyzer
- 100 µl of PT reagent(Thromborel-S) was added

Results in the display were read – it gave the PT value in sec.

Activated Partial Thromboplastin Time (APTT) Principle

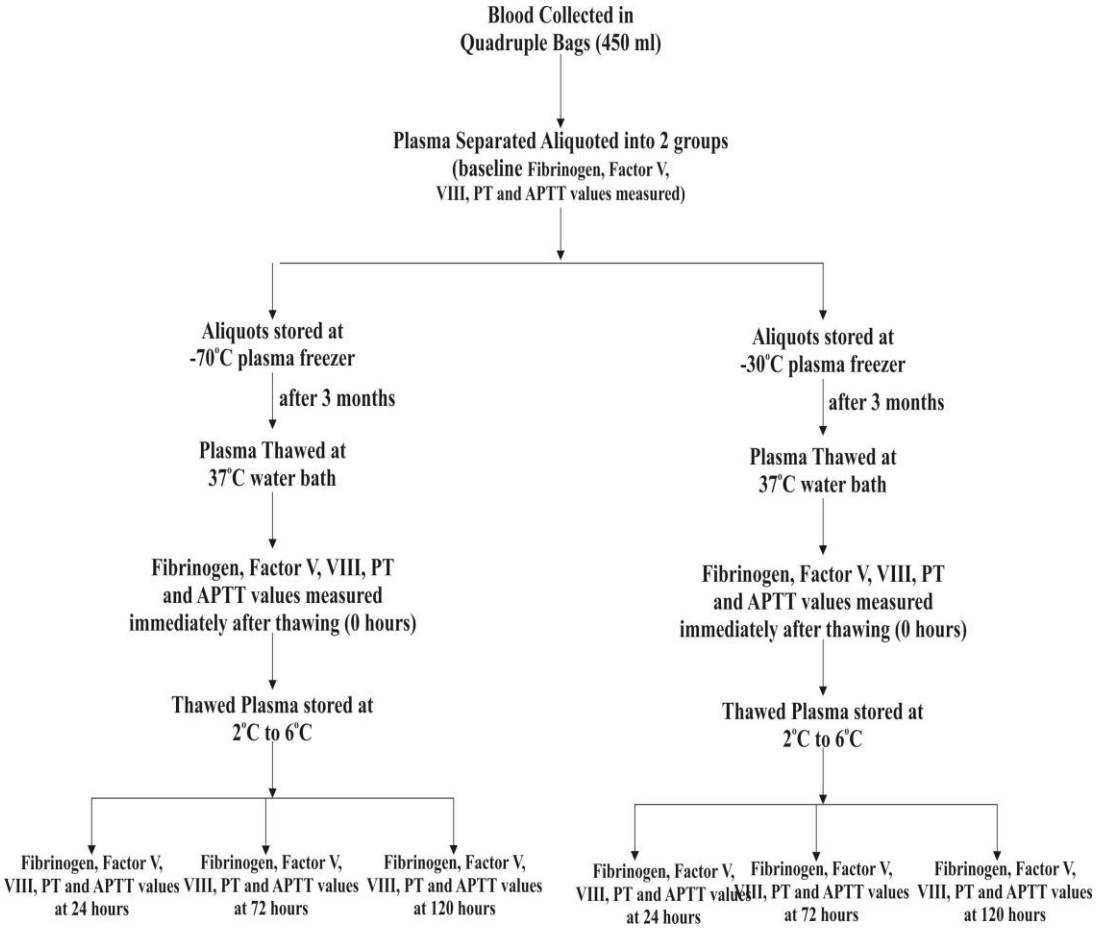
The test measures the clotting time of plasma on addition of phospholipid and calcium chloride and avoiding tissue thromboplastin. It activates contact factors and shows the functional status of the coagulation factors involved in intrinsic pathway.

Method

- 50 µl of plasma was taken in a test cuvette
- 50 µl of Erba Actime reagent was added
- It was incubated it for 2 minutes as programmed in Erba ECL 412 analyzer
- 50µl Calcium Chloride (0.025M $CaCl_2$) was added

The results in the display were read – it gave the APTT value in sec.

METHODOLOGY FLOWCHART



Statistical analysis

- All statistical analysis was performed using Statistical Package for Social Science (SPSS, version 17) for Microsoft windows.

- The data were normally distributed and therefore parametric tests were performed.

- The data were expressed as Mean and SD.

- Independent sample student t test were used to compare continuous variables between two groups. Paired sample test were used for within groups. A two sided p value < 0.05 was considered statistically significant.

RESULTS

In both the groups, even the coagulation values measured immediately after thawing (day 0) showed a statistically significant difference compared to baseline. This significance extended up to day 5. However, all the values of observed parameters were within the physiological limits. This shows that the process of storage of Fresh Frozen Plasma does cause a decrease in the coagulation factors immaterial of the storage temperature, though within therapeutic range.

Between the groups, the -70°C group performed better in retaining the stability of coagulation factors. This was evident in the statistically significant difference observed between the groups at 72 hours and 120 hours after thawing and storage at 2 to 6°C. The values observed in the -30°C group was however in the hemostatic range.

Among the factors, fibrinogen was found to be the most stable with lesser fluctuations from the baseline values. Factor VIII was observed to be the most labile among the observed parameters, decreasing by 28% from the baseline value. Yet, the therapeutic measure of atleast 0.5 units/ml as suggested by the European Pharmacopoeia was not breached.

Table 1: Comparison of fibrinogen levels at -30°C and -70°C

	Group	N	Mean	Std. Deviation	Sig
FIB mg/dl – Baseline	-30°C	40	289.1625	10.72169	1.00
	-70°C	40	289.1625	10.72169	
FIB mg/dl – 0 hours	-30°C	40	283.2200	10.11078	0.479
	-70°C	40	284.8400	10.27908	
FIB mg/dl – 24 hours	-30°C	40	277.7350	9.77494	0.204
	-70°C	40	280.5975	10.18930	
FIB mg/ml – 72 hours	-30°C	40	271.3050	9.71100	0.040*
	-70°C	40	275.8600	9.84117	
FIB mg/dl – 120 hours	-30°C	40	264.3325	9.60837	0.004**
	-70°C	40	270.8350	10.06554	

*P<0.05, ** p<0.01, N = No. of samples

There is statistical significant difference between Sample -30°C and Sample -70°C at 72 Hours and 120 Hours in fibrinogen levels.

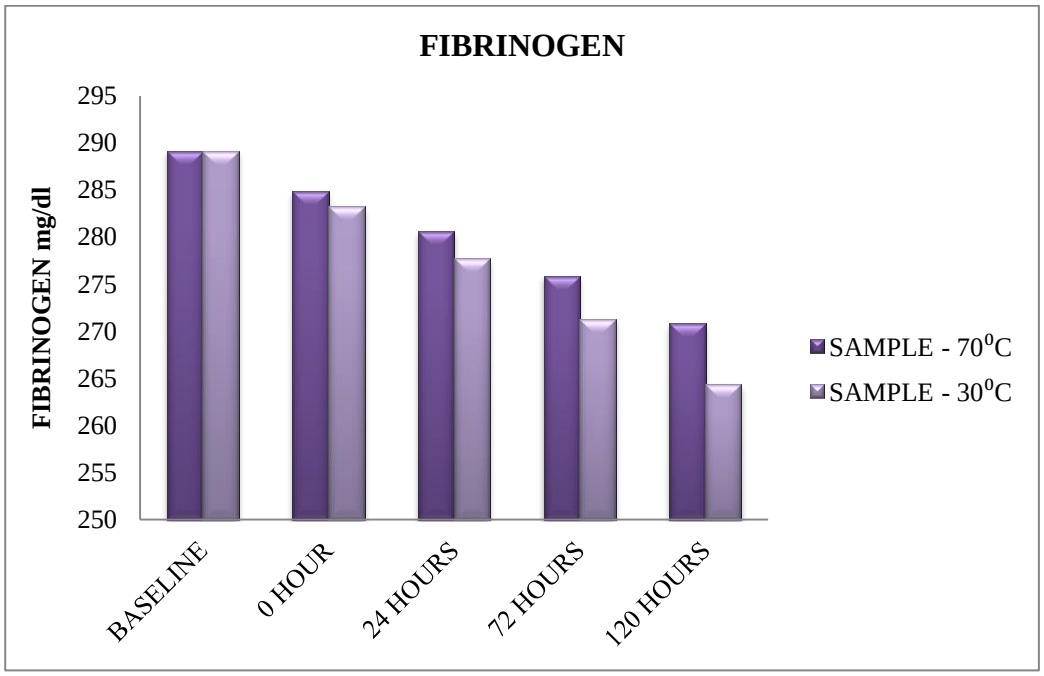


Fig.5: Comparison of fibrinogen levels at -30°C and -70°C

Table 2: Comparison of Factor V levels at -30°C and -70°C

	Group	N	Mean	Std. Deviation	Sig
F V IU/ml - Baseline	-30°C	40	.9957	.11953	1.00
	-70°C	40	.9957	.11953	
F V IU/ml - 0 hour	-30°C	40	.9175	.09111	0.181
	-70°C	40	.9475	.10703	
F V IU/ml - 24 hours	-30°C	40	.8525	.08602	0.071
	-70°C	40	.8895	.09484	
F V IU/ml - 72 hours	-30°C	40	.7763	.07427	0.009**
	-70°C	40	.8238	.08307	
FV IU/ml - 120 hours	-30°C	40	.6948	.06421	0.000***
	-70°C	40	.7573	.07961	

**P<0.01, *** p<0.001

There is statistical significant difference between Sample at -30°C and Sample at -70°C at 72 Hours and 120 Hours in Factor V levels.

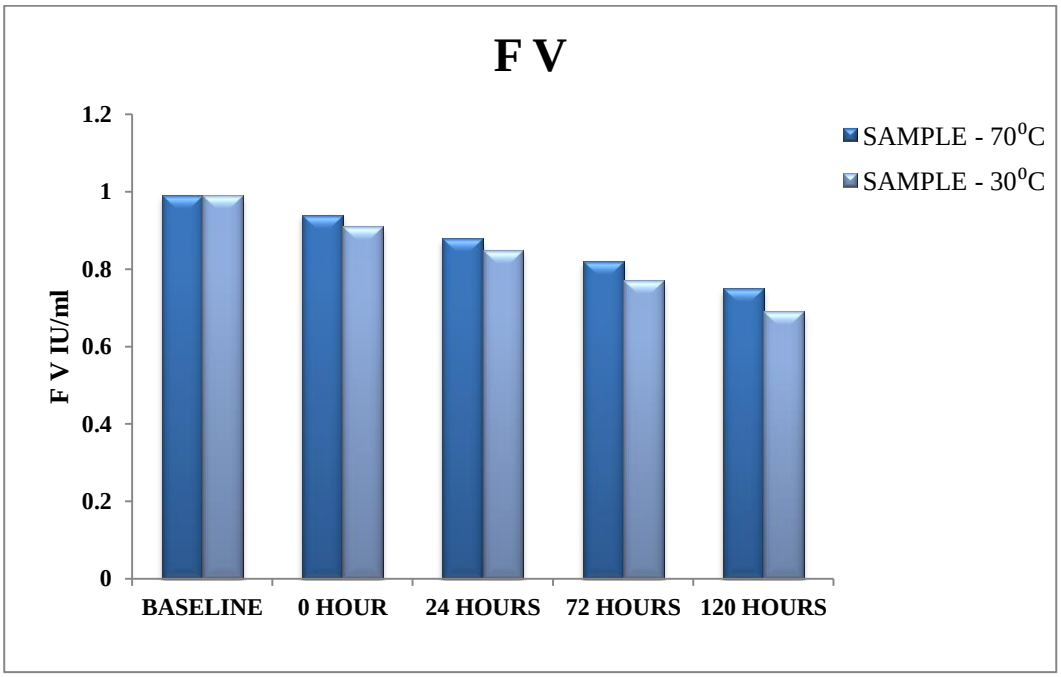


Fig.6: Comparison of Factor V levels at -30°C and -70°C

Table 3: Comparison of Factor VIII levels at -30°C and -70°C

	Group	N	Mean	Std. Deviation	Sig
F VIII IU/ml – baseline	-30°C	40	.9348	.07524	1.00
	-70°C	40	.9348	.07524	
F VIII IU/ml – 0 hour	-30°C	40	.8507	.06738	0.097
	-70°C	40	.8760	.06686	
F VIII IU/ml – 24 hours	-30°C	40	.7675	.06905	0.154
	-70°C	40	.7885	.06133	
F VIII IU/ml – 72 hours	-30°C	40	.6793	.06154	0.010**
	-70°C	40	.7163	.06356	
F VIII IU/ml – 120 hours	-30°C	40	.5822	.05299	0.002**
	-70°C	40	.6232	.05963	

**P<0.01

There is statistical significant difference between Sample -30°C and Sample -70°C at 72 Hours and 120 Hours in F VIII levels.

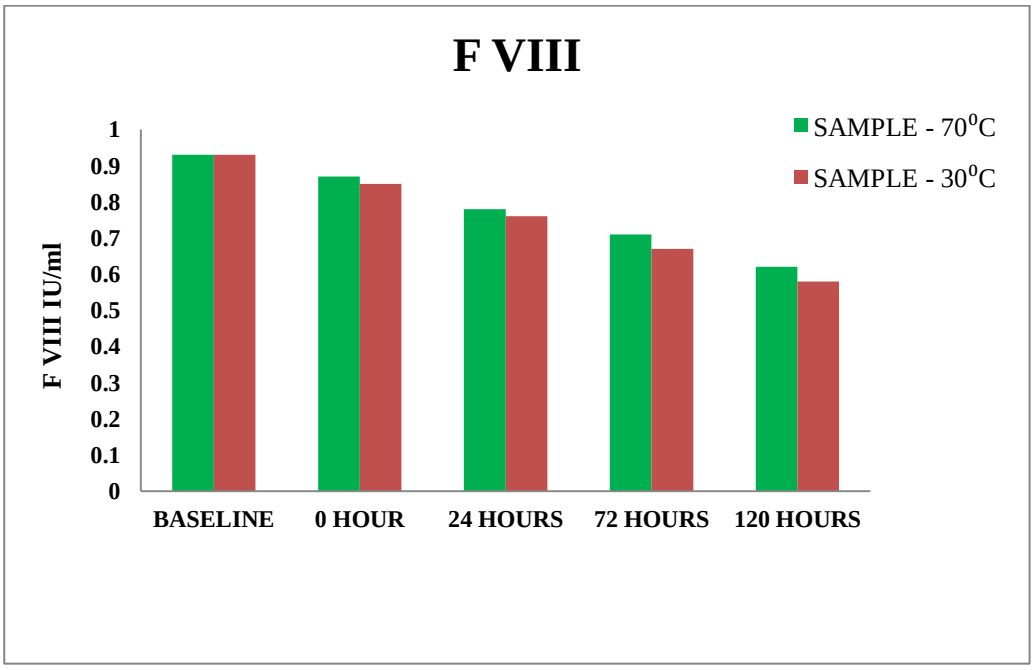


Fig.7: Comparison of Factor VIII levels at -30°C and -70°C

Table 4: Comparison of Prothrombin time at -30°C and -70°C

	Group	N	Mean	Std. Deviation	Sig
PT secs - baseline	-30°C	40	13.473	.9457	1.00
	-70°C	40	13.473	.9457	
PT secs – 0 hour	-30°C	40	13.980	.9595	0.297
	-70°C	40	13.755	.9565	
PT secs - 24 hours	-30°C	40	14.468	.9638	0.221
	-70°C	40	14.198	9940	
PT secs – 72 hours	-30°C	40	15.03	.949	0.567
	-70°C	40	14.90	.959	
PT secs – 120 hours	-30°C	40	15.95	.955	0.695
	-70°C	40	15.87	.922	

There is no statistical significant difference between Sample -30°C and Sample -70°C in PT at p> 0.05.

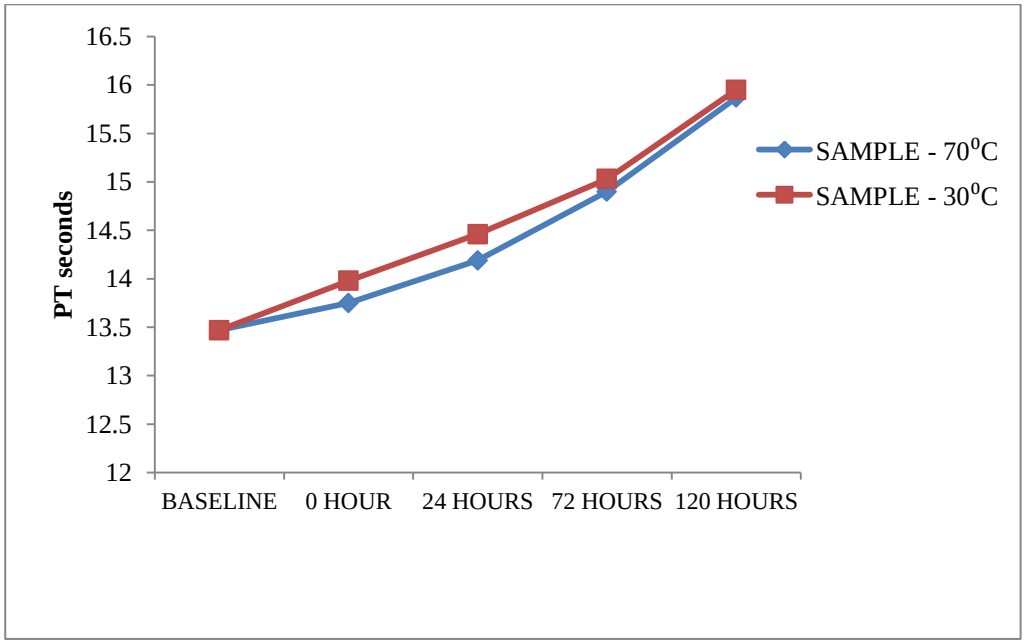


Fig.8: Comparison of Prothrombin time at -30°C and -70°C

Table 5: Comparison of Activated partial thromboplastin time at -30°C and -70°C

	Group	N	Mean	Std. Deviation	Sig
APTT secs - baseline	-30°C	40	29.148	1.3204	1.00
	-70°C	40	29.148	1.3204	
APTT secs - 0 hour	-30°C	40	29.750	1.3044	0.457
	-70°C	40	29.530	1.3259	
APTT secs - 24 hours	-30°C	40	30.99	1.306	0.127
	-70°C	40	30.54	1.346	
APTT secs - 72 hours	-30°C	40	33.023	1.2589	0.112
	-70°C	40	32.563	1.3006	
APTT secs - 120 hours	-30°C	40	34.073	1.2876	0.155
	-70°C	40	33.658	1.2969	

There is no statistical significant difference between Sample -30°C and Sample -70°C in APTT at $p > 0.05$

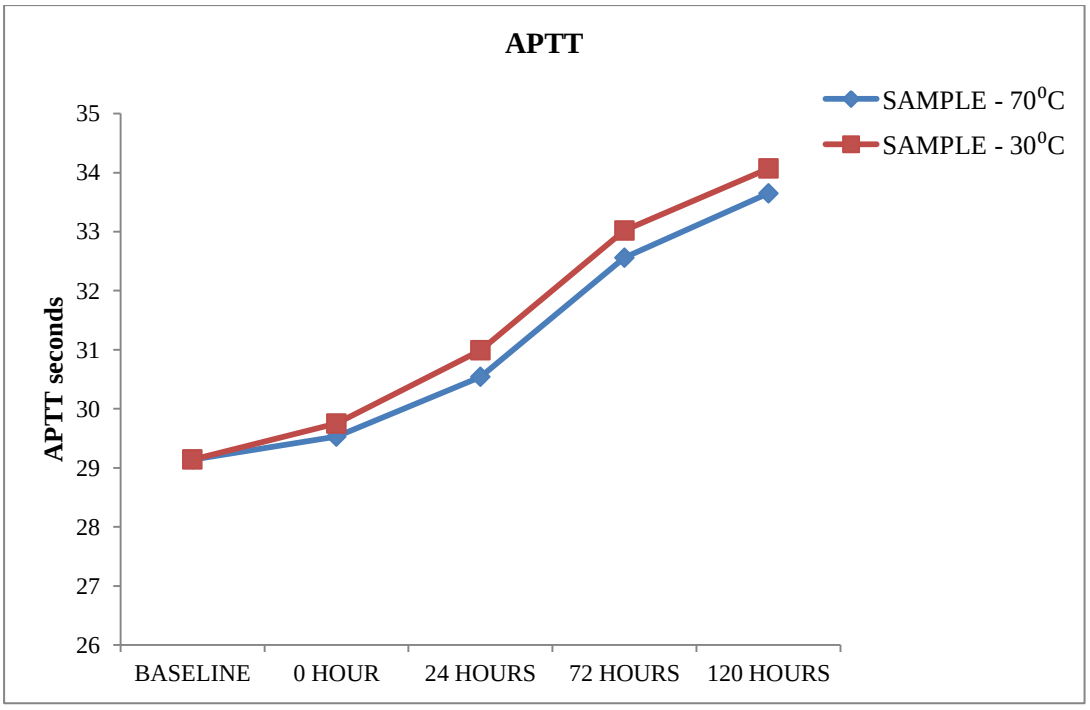


Fig.9: Comparison of Activated partial thromboplastin time at -30°C and -70°C

Table 6: Comparison between baseline and follow up in fibrinogen at -30° C

		Mean	N	Std. Deviation	Sig
Pair 1	FIB mg/dl - baseline	289.1625	40	10.72169	0.000***
	FIB mg/dl - 0 hour	283.2200	40	10.11078	
Pair 2	FIB mg/dl - baseline	289.1625	40	10.72169	0.000***
	FIB mg/dl - 24 hours	277.7350	40	9.77494	
Pair 3	FIB mg/dl - baseline	289.1625	40	10.72169	0.000***
	FIB mg/ml - 72 hours	271.3050	40	9.71100	
Pair 4	FIB mg/dl - baseline	289.1625	40	10.72169	0.000***
	FIB mg/dl - 120 hours	264.3325	40	9.60837	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24, 72 and 120 hours in Sample -30°C.

Table 7: Comparison between baseline and follow up in fibrinogen at -70° C

		Mean	N	Std. Deviation	Sig
Pair 1	FIB mg/dl - baseline	289.1625	40	10.72169	0.000***
	FIB mg/dl - 0 hour	284.8400	40	10.27908	
Pair 2	FIB mg/dl - baseline	289.1625	40	10.72169	0.000***
	FIB mg/dl - 24 hours	280.5975	40	10.18930	
Pair 3	FIB mg/dl - baseline	289.1625	40	10.72169	0.000***
	FIB mg/ml - 72 hours	275.8600	40	9.84117	
Pair 4	FIB mg/dl - baseline	289.1625	40	10.72169	0.000***
	FIB mg/dl - 120 hours	270.8350	40	10.06554	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24, 72 and 120 hours in Sample -70°C

Table 8: Comparison between baseline and follow up in F V at -30°C

		Mean	N	Std. Deviation	Sig
Pair 1	FV IU/ml - baseline	.9957	40	.11953	0.000***
	FV IU/ml - 0 hour	.9175	40	.09111	
Pair 2	FV IU/ml - baseline	.9957	40	.11953	0.000***
	FV IU/ml - 24 hours	.8525	40	.08602	
Pair 3	FV IU/ml - baseline	.9957	40	.11953	0.000***
	FV IU/ml - 72 hours	.7763	40	.07427	
Pair 4	FV IU/ml - baseline	.9957	40	.11953	0.000***
	FV IU/ml - 120 hours	.6948	40	.06421	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24, 72 and 120 hours in Sample -30°C.

Table 9: Comparison between baseline and follow up in F V at -70°C

		Mean	N	Std. Deviation	Sig
Pair 1	FV IU/ml - baseline	.9957	40	.11953	0.000***
	FV IU/ml - 0 hour	.9475	40	.10703	
Pair 2	FV IU/ml - baseline	.9957	40	.11953	0.000***
	FV IU/ml - 24 hours	.8895	40	.09484	
Pair 3	FV IU/ml - baseline	.9957	40	.11953	0.000***
	FV IU/ml - 72 hours	.8238	40	.08307	
Pair 4	FV IU/ml - baseline	.9957	40	.11953	0.000***
	FV IU/ml - 120 hours	.7573	40	.07961	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24, 72 and

Table 10: Comparison between baseline and follow up in F VIII at -30°C

		Mean	N	Std. Deviation	Sig
Pair 1	FVIII IU/ml - baseline	.9348	40	.07524	0.000***
	FVIII IU/ml - 0 hour	.8507	40	.06738	
Pair 2	FVIII IU/ml - baseline	.9348	40	.07524	0.000***
	FVIII IU/ml - 24 hours	.7675	40	.06905	
Pair 3	FVIII IU/ml - baseline	.9348	40	.07524	0.000***
	FVIII IU/ml - 72 hours	.6793	40	.06154	
Pair 4	FVIII IU/ml - baseline	.9348	40	.07524	0.000***
	FVIII IU/ml - 120 hours	.5822	40	.05299	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24,72 and 120 hours in Sample -30°C.

Table 11: Comparison between baseline and follow up in F VIII at -70°C

		Mean	N	Std. Deviation	Sig
Pair 1	FVIII IU/ml - baseline	.9348	40	.07524	0.000***
	FVIII IU/ml - 0 hour	.8760	40	.06686	
Pair 2	FVIII IU/ml - baseline	.9348	40	.07524	0.000***
	FVIII IU/ml - 24 hours	.7885	40	.06133	
Pair 3	FVIII IU/ml - baseline	.9348	40	.07524	0.000***
	FVIII IU/ml - 72 hours	.7163	40	.06356	
Pair 4	FVIII IU/ml - baseline	.9348	40	.07524	0.000***
	FVIII IU/ml - 120 hours	.6232	40	.05963	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24,72 and 120 hours in Sample -70°C.

Table 12: Comparison between baseline and follow up in PT at -30°C

		Mean	N	Std. Deviation	Sig
Pair 1	PT - baseline	13.473	40	.9457	0.000***
	PT - 0 hours	13.980	40	.9595	
Pair 2	PT - baseline	13.473	40	.9457	0.000***
	PT - 24 hours	14.468	40	.9638	
Pair 3	PT - baseline	13.473	40	.9457	0.000***
	PT - 72 hours	15.03	40	.949	
Pair 4	PT - baseline	13.473	40	.9457	0.000***
	PT - 120 hours	15.95	40	.955	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24,72 and 120 hours in Sample -30°C.

Table 13: Comparison between baseline and follow up in PT at -70°C

		Mean	N	Std. Deviation	Sig
Pair 1	PT - baseline	13.473	40	.9457	0.000***
	PT - 0 hours	13.755	40	.9565	
Pair 2	PT - baseline	13.473	40	.9457	0.000***
	PT - 24 hours	14.198	40	.9940	
Pair 3	PT - baseline	13.473	40	.9457	0.000***
	PT - 72 hours	14.90	40	.959	
Pair 4	PT - baseline	13.473	40	.9457	0.000***
	PT - 120 hours	15.87	40	.922	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24, 72 and 120 hours in Sample -70°C.

Table 14: Comparison between baseline and follow up in APTT at -30°C

		Mean	N	Std. Deviation	Sig
Pair 1	APTT - baseline	29.148	40	1.3204	0.000***
	APTT - 0 hours	29.750	40	1.3044	
Pair 2	APTT - baseline	29.148	40	1.3204	0.000***
	APTT - 24 hours	30.99	40	1.306	
Pair 3	APTT - baseline	29.148	40	1.3204	0.000***
	APTT - 72 hours	33.023	40	1.2589	
Pair 4	APTT - baseline	29.148	40	1.3204	0.000***
	APTT - 120 hours	34.073	40	1.2876	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24,72 and 120 hours in Sample -30°C.

Table 16: Comparison between baseline and follow up in APTT at -70°C

		Mean	N	Std. Deviation	Sig
Pair 1	APTT - baseline	29.148	40	1.3204	0.000***
	APTT - 0 hours	29.530	40	1.3259	
Pair 2	APTT - baseline	29.148	40	1.3204	0.000***
	APTT - 24 hours	30.54	40	1.346	
Pair 3	APTT - baseline	29.148	40	1.3204	0.000***
	APTT - 72 hours	32.563	40	1.3006	
Pair 4	APTT - baseline	29.148	40	1.3204	0.000***
	APTT - 120 hours	33.658	40	1.2969	

*** $p < 0.001$

There is statistical significant difference between baseline and 0, 24,72 and 120 hours in Sample -70°C

CORRELATION

Table 16: Correlation between APTT and F VIII at -70^{0}C

	APTT at -70^{0}C	F VIII at -70^{0}C
Baseline	29.14	0.93
0 hour	29.53	0.87
24 hours	30.54	0.78
72 hours	32.56	0.71
120 hours	33.65	0.62

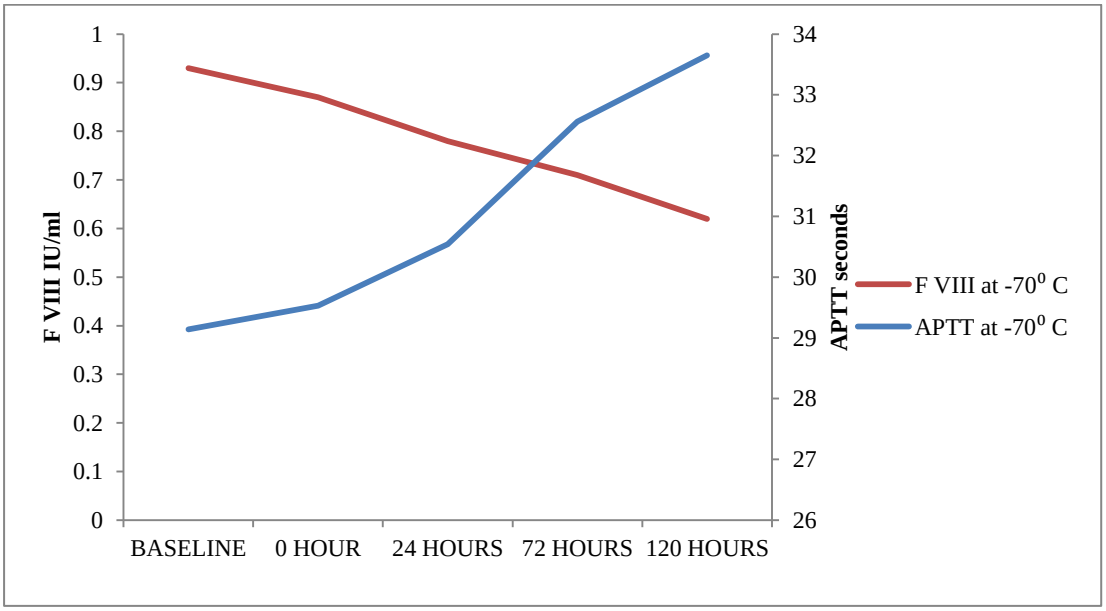


Fig.10: Correlation between APTT and F VIII at -70^{0}C

Table 17: Correlation between APTT and F VIII at -30^{0}C

	APTT at -30^{0}C	F VIII at -30^{0}C
Baseline	29.14	0.93
0 hour	29.75	0.85
24 hours	30.99	0.76
72 hours	33.02	0.67
120 hours	34.07	0.58

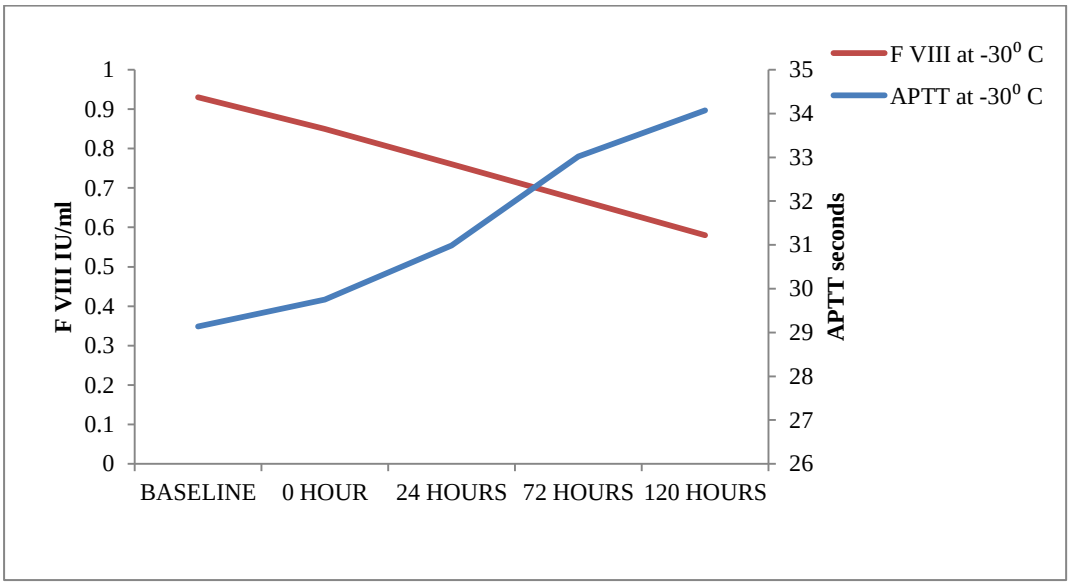


Fig.11: Correlation between APTT and F VIII at -30^{0}C

Table 18: Correlation between fibrinogen and PT at -70^{0}C

	PT at -70^{0}C	Fibrinogen at -70^{0}C
Baseline	13.47	289.16
0 hour	13.75	284.84
24 hours	14.19	280.59
72 hours	14.9	275.86
120 hours	15.87	270.83

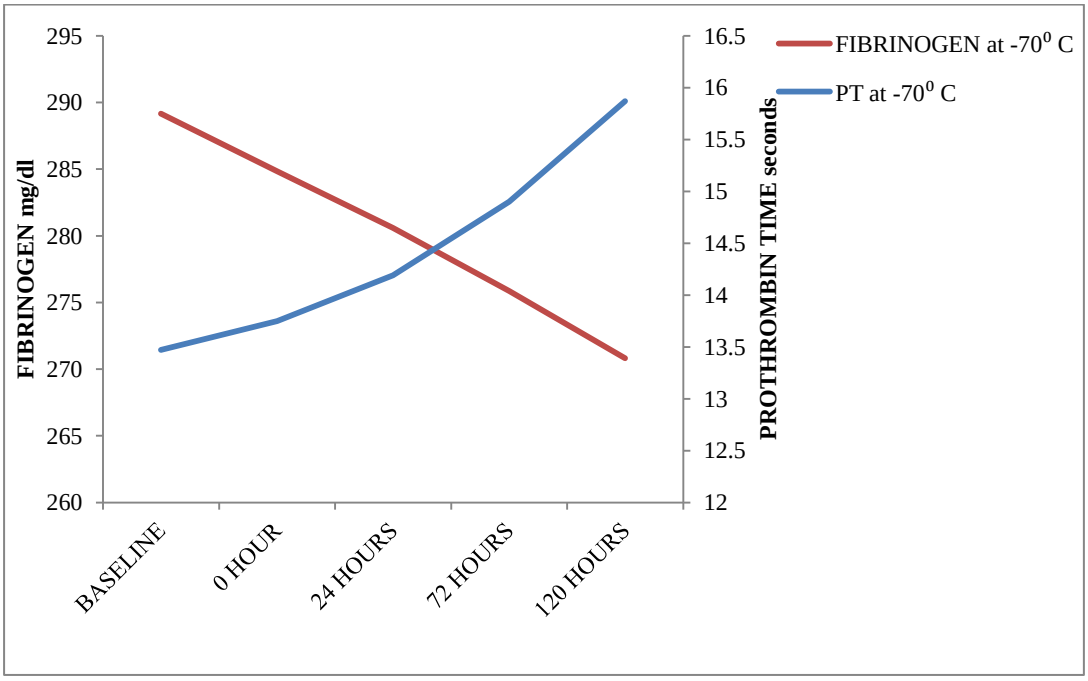


Fig.12: Correlation between fibrinogen and PT at -70^{0}C

Table 19: Correlation between fibrinogen and PT at -30^{0}C

	PT at -30^{0}C	Fibrinogen at -30^{0}C
Baseline	13.47	289.16
0 hour	13.98	283.22
24 hours	14.46	277.73
72 hours	15.03	271.3
120 hours	15.95	264.33

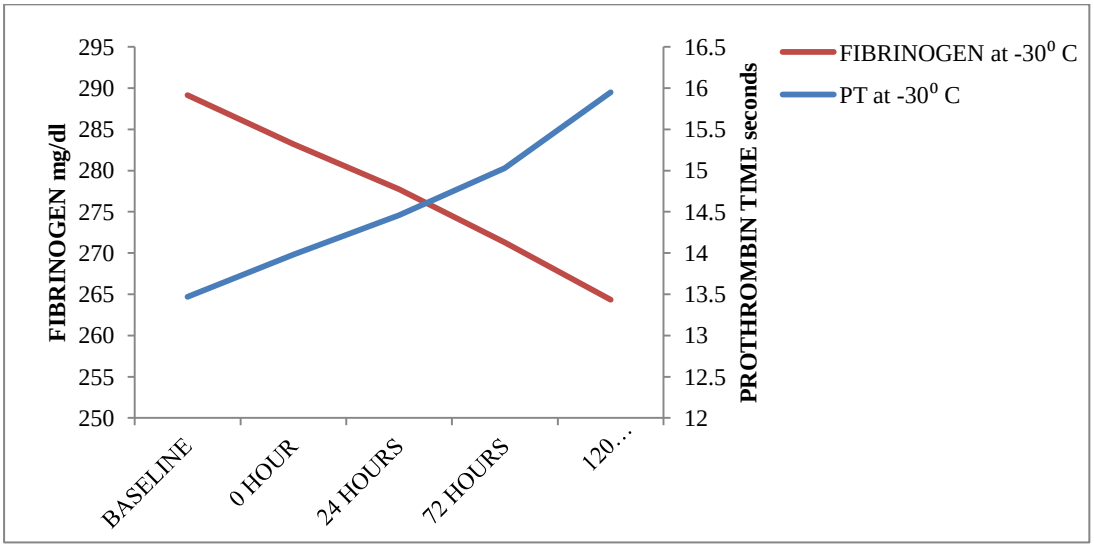


Fig.13: Correlation between fibrinogen and PT at -30^{0}C

Table 20: Correlation between F V, PT and APTT at -70^{0}C

	PT at -70^{0}C	APTT at -70^{0}C	F V at -70^{0}C
Baseline	13.47	29.14	0.99
0 hour	13.75	29.53	0.94
24 hours	14.19	30.54	0.88
72 hours	14.9	32.56	0.82
120 hours	15.87	33.65	0.75

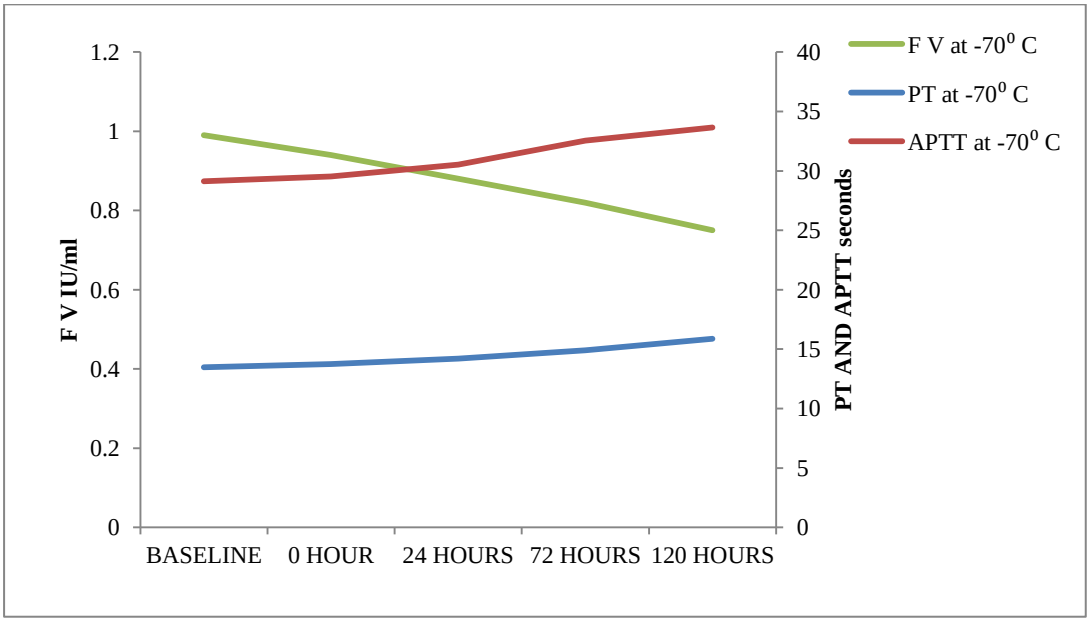


Fig.14: Correlation between F V, PT and APTT at -70^{0}C

Table 21: Correlation between F V, PT and APTT at -30^{0}C

	PT at -30^{0}C	APTT at -30^{0}C	F V at -30^{0}C
Baseline	13.47	29.14	0.99
0 hour	13.98	29.75	0.91
24 hours	14.46	30.99	0.85
72 hours	15.03	33.02	0.77
120 hours	15.95	34.07	0.69

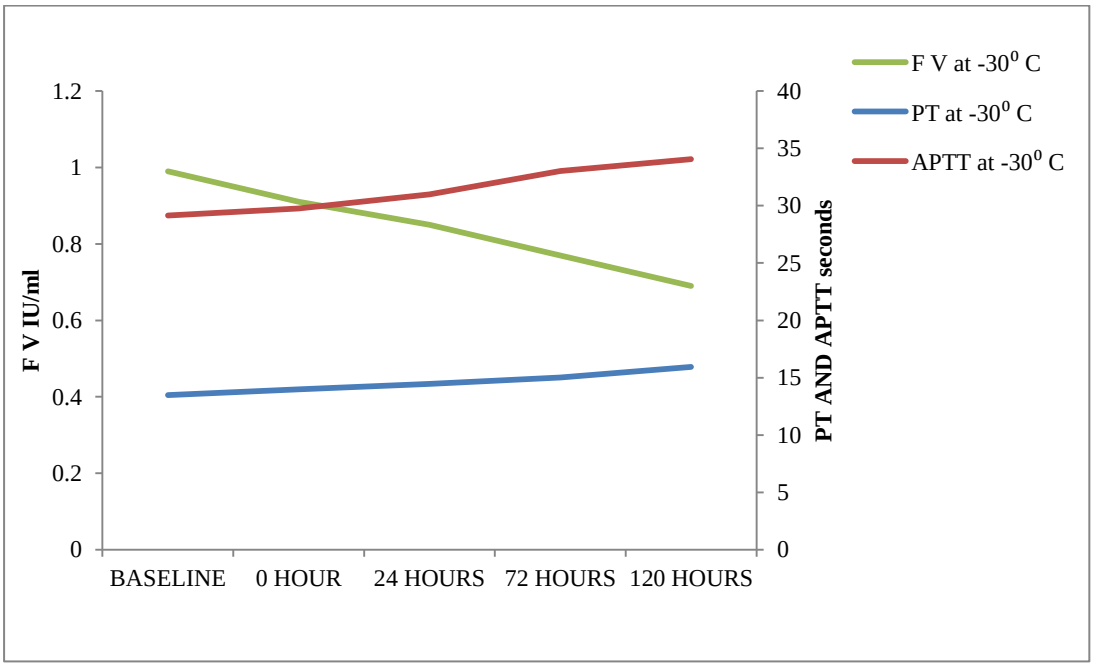


Fig.15: Correlation between F V, PT and APTT at -30^{0}C

DISCUSSION

FFP is an important derivative of either whole blood or apheresed blood. It finds a multitude of applications in routine critical care and intra-operative settings. A prolonged shelf life of thawed plasma implies greater utility and decreased wastage. This should however be accomplished with retention of the functional properties of FFP. A number of studies and audits have been done worldwide to analyse this requirement.[73,74,75]

In a country like ours with limited resources, optimal usage of available facilities and materials should be done. These available resources for storage of FFP vary from center to center. This prompted us to perform this study. Through this study we aimed to compare the stability of coagulation factors in FFP that was stored previously at -30°C and at -70°C.

Blood samples were collected from the department of Transfusion Medicine and from Government Kilpauk Medical College which is a CEmONC (Comprehensive Emergency Obstetrics and Newborn Care) centre. The baseline values of the observed parameters were measured immediately after separation of FFP. These FFP were divided into two groups . One set was frozen at -70°C and another set was frozen at -30°C.

In our study, Prothrombin time (PT) values were prolonged by 0.28 seconds immediately after thawing and by 2.4 seconds at 120 hours in -70°C group compared to baseline values. Similarly, the -30℃ group also showed an increase of 0.51 seconds after thawing and 2.48 seconds over the period of 5 days. The changes were statistically significant even as early as 0 hours of thawing in both the groups. However, the values were within the therapeutic range in both the groups over the

period of analysis and the difference between the groups was not statistically significant.

Nagadeh HT[62] performed a similar study except that the freezing process of plasma was to a single temperature by blast freezer. They observed that the average lengthening of Prothrombin time was statistically significant from day 1 to day 5 but was within the physiological limits. Their results correlate with our findings. A similar observation has been made by Yazer MH[27] and associates. They found that the percentage increase in PT from day 0 to day 5 was 22% which is identical to the increase observed by us. Both the above mentioned authors demonstrated that though there is a modest increase in PT values on storage of thawed plasma, it does not exceed the permissible limits and our study too complies with theirs.

Activated Partial Thromboplastin Time (aPTT) values too showed a trend similar to the PT values in our study. There was an increase of 15.47% from baseline at day 5 in -70°C group and 16.91% in -30°C group. Though the increase was statistically significant within the group, the inter group variations were not statistically significant. The increase was also not pathological.

An increase in APTT values by 12.2% from day 1 to day 5 has been observed by Nagadeh HT and colleagues.[62] We too met with a similar trend in our study. The -70°C group showed an increase by 13.95% from day 1 to day 5 and the -30°C group, by 14.52% over the duration of storage. Yazer MH and colleagues[27] too showed a similar result wherein the increase in APTT values was 10.3%. They had opined that APTT values are in the therapeutic range at the end of 5 days of thawed plasma storage at 1°C to 6°C.

Fibrinogen levels showed a decrease on storage of thawed plasma at 2°C to 6°C over 120 hours. The degree of fall was by 6.33% in -70°C group and 8.58% in

-30°C group. Both the groups showed statistical significant difference at 72 hours and 120 hours of storage with respect to the change in fibrinogen levels between them.

Noordin SS and friends[71] published a study in Indian Journal Of Haematology and Blood Transfusion in the year 2017 and they observed a fall in fibrinogen levels by 8.01% at the end of day 5 when compared to the baseline value and this is identical to our findings. Further, a statistically significant change even on day 0 compared to baseline values was recorded by them as well as by us.

In contrast, Sidhu RS and associates[76] in 2006 have demonstrated a non-significant change of 1.9% in fibrinogen levels from day 1 to day 5 in the research article in Journal of Clinical Apheresis, whereas we recorded a significant change of 6.66% and 4.91% in -30℃ and -70°C groups respectively from day 1 to day 5.

Gosselin RC and colleagues[68] in 2015 have found that fibrinogen values are the least affected by vial types, freezing and thawing conditions and temperatures. Earlier, Alesci S and others have also demonstrated that freezing and storage methods have minimal or no effect on fibrinogen assays in their publication in Thrombosis Research in 2009.

Downes KA[77] also found no decrease in fibrinogen levels on storage of thawed and stored plasma over a period of 5 days.

The degree of fall in Factor V levels on storage was statistically significant within the individual groups (24.24% in -70°C group vs 30.30% in -3°C group). In comparison to the baseline values, the levels in the samples immediately after thawing (0 hour) also showed a statistically significant fall in both the groups and it continued till 120 hours. When the changes were compared between the groups, it was observed that the -30°C group had a more significant fall in F V levels than

-70°C group from 72 hours onwards. The clinical outcome of the fall however was not of significance because the coagulation tests were within permissible limits.

Buchta C[52] and others in the year 2004 published their findings in Vox Sanguinis and they observed a fall in F V levels to 78% of baseline values on day 5 of storage of thawed plasma at 4℃. This is similar to the 24% fall reported in our study.

Smak Gregoor PJ et al,[50] way back in 1993 have analysed the coagulation factor levels of FFP and cryoprecipitate free plasma. They observed that the levels of F V decreased by up to 36% in FFP group and by 42% in the cryoprecipitate free plasma group at the end of 28 days of storage at 4℃. They further added that such stored plasma over 28 days retained the properties to achieve adequate hemostasis.

Lamboo M and colleagues[70] performed an identical study in Netherlands and published their recordings in Transfusion Medicine in 2007. Their results showed that the F V level decreased by 35% at the end of 2 weeks of storage of thawed plasma and this fall was gradual from the baseline values. They reported that once thawed, FFP can be stored in room temperature for 6 hours and up to 2 weeks at 4°C.

Nagadeh HT and others[62] too observed a fall of 20% in day 5 of storage from day 1.

A still higher fall of 33.5% from day 1 was reported by Yazer MH and friends[27] in their study published in Transfusion in 2008. Both the above authors however said that the values were within therapeutic range.

Our findings correlate with the above mentioned authors.

In contrast, Downes KA et al[77] observed an insignificant fall of 16% in F V levels on day 5 relative to day 1. Their study also opined that the fall does not affect the clinical outcome of the analysed samples and the stored plasma can be safely administered to patients in need.

Sidhu RS and colleagues[76] reported a lesser degree of fall of 8.8% of F V levels over the study duration but the change was statistically significant.

Factor VIII was found to be the most labile factor in our study. Both the groups showed a significant reduction in the values of F VIII as early as immediately after thawing in comparison to the baseline values over the study period. The inter group difference was also statistically significant. Though the fall at the end of day 5 was by 37% in -30℃ group, the absolute value of 0.5822 was above minimal requirement of 0.5 units/ml recommended by the European Pharmacopoeia.[78] Yet, the clinical results in the form of APTT did not reflect any deviation from the physiological limits.

Buchta C[52] and colleagues obtained a reduction of 22% in F VIII values on storage and the end values were within the treatment requisites. This is similar to the 28% observed by us in the -70°C group. However, the -30°C group had a more pronounced fall in F VIII levels by 31.58% in our study.

Downes KA[77] had reported in their study that the fall in F VIII levels over the period of storage represents a true decline in the coagulation factors. They observed a 41% fall in groups A and O and 35% fall in group B. Their findings correlate with our observations. However they have summarized that thawed and stored plasma can be used to treat patients with coagulopathy and not for patients with isolated F VIII deficiencies.

Sidhu RS[76] and others observed a fall of 14.3% in F VIII levels over the 5 day period. This was lesser than the recordings seen in our study. However the end point values were within the recommended therapeutic requisites.

In contrast, Noordin SS et al[71] reported a greater fall (>50%) in F VIII levels on storage of thawed plasma. Their values decreased from 71.6 to 35.9 at the end of 5 days. They have opined that the greater degree of fall could be because of the lower initial values at the start of the analysis.

According to DGHS,[19] once thawed the FFP should be administered at the earliest and maximum within 24 hours if stored at 2°C to 6°C.

According to AABB,[36] FFP on thawing has a shelf life of 24 hours at 1°C to 6°C. FFP which is thawed and stored longer than 24 hours must be relabeled as Thawed Plasma, and it can be stored for an additional 4 days at 1°C to 6°C. In our study as mentioned above we have studied the extended shelf life of plasma after thawing and storing at 2°C to 6°C for 5 days.

Numerous studies have been done to analyse the probable storage time of thawed plasma frozen at various temperatures and stored over various duration. We have compared the results of those studies with ours.

COMPARISON OF OUR STUDY WITH OUTCOME OF OTHER STUDIES

Changes in coagulation factors in thawed and stored plasma at 2°C to 6°C for 5 days

S. No	Parameters	Our study	Nagadeh et al	Sidhu et al	Yazer et al	Lamboo et al	Noordin et al	Downes et al	Gregoor et al
1	Factor V	Decreased by 20.07%	Decreased by 20%	Decreased by 8.8%	Decreased by 33.5%	Decreased by 35%	Decreased by 20.8%	Decreased by 16%	Decreased by 36%
2	Factor VIII	Decreased by 28.85%	Decreased by 25%	Decreased by 14.3%	Decreased by 2.69%	Decreased by 45%	Decreased by 48.46%	Decreased by 41%	Decreased by 36%
3	Fibrinogen	Decreased by 4.91%	-	Decreased by 1.9%	Decreased by 1.66%	Decreased by 8%	Decreased by 8.01%	0% change	Decreased by 7.8%
4	PT	Prolonged by 15.4%	Prolonged by 8.8%	-	Prolonged by 22%	Prolonged by 15%	Prolonged by 16.6%	-	Prolonged by 22.68%
5	APTT	Prolonged by 12.24%	Prolonged by12.2%	-	Prolonged by 10.3%	Prolonged by 17%	Prolonged by 10.3%	-	Prolonged by 32%

SUMMARY

In our study, we evaluated the stability of coagulation factors V and VIII on freezing plasma at different temperatures. We also analysed the levels of factors on storing thawed plasma at 2°C to 6°C for 5 days.

- 40 units were separated in to 2 aliquots and stored at -30°C and -70°C. At the end of 3 months, these samples were thawed and evaluated for factor V, VIII, fibrinogen, PT and APTT on day 0. Then, the thawed plasma samples were stored at 2°C to 6°C, which were evaluated for the above parameters on day 1, 3 & 5. The values for the above parameters were also noted on the day of collection.

- The values on the day of collection for 40 samples were:

    - Fibrinogen – 289.16 mg/dl (268.4 – 314.8 mg/dl)
    - F V - 0.99 IU/ml (0.83 – 1.32 IU/ml)
    - F VIII - 0.93 IU/ml (0.83 – 1.17 IU/ml)
    - PT – 13.47 seconds (12.1 – 14.9 seconds)
    - APTT – 29.14 seconds (27.5 – 35.0 seconds)

The value on the subsequent days were as follows:

Day	Storage Temperature	Fibrinogen mg/dl	F V IU/ml	F VIII IU/ml	PT seconds	APTT seconds
Day 0	-30^{0}C	283.22	0.91	0.85	13.98	29.75
	-70^{0}C	284.84	0.94	0.87	13.75	29.53
Day1	-30^{0}C	277.73	0.85	0.76	14.46	30.99
	-70^{0}C	280.59	0.88	0.78	14.19	30.54
Day3	-30^{0}C	271.3	0.77	0.67	15.03	33.02
	-70^{0}C	275.86	0.82	0.71	14.9	32.56
Day5	-30^{0}C	264.33	0.69	0.58	15.95	34.07
	-70^{0}C	270.83	0.75	0.62	15.87	33.65

Interpolation

- From Day 0 to Day 5 the plasma clotting factors-Fibrinogen, Factor V and Factor VIII levels are decreasing gradually on storing at 2°C to 6°C .Simultaneously PT and APTT values are increasing as we mentioned in the above table.

- It showed the correlation between plasma clotting factor Fibrinogen and PT value, factor VIII and APTT value, Factor V and PT, APTT values.

CONCLUSION

In our study, the coagulation factors V, VIII and fibrinogen values were essentially within accepted therapeutic range on day 5 of thawed plasma stored at 2 to 6°C, even though there was a statistically significant difference observed. This enables reduced wastage of unused thawed plasma with qualitatively acceptable factor levels. In emergency situations, these units can be issued and utilized immediately as a life saving measures.

Further, our study showed acceptable correlation between PT, APTT values and coagulation factor levels, which approximately evaluates the quality of plasma in centres lacking facilities to do factor assay.

This study reiterates the extended shelf-life of thawed plasma, which can be utilized in emergency situations in CEmONC and tertiary care hospitals.

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PLAGIARISM CERTIFICATE - II

This is to certify that this dissertation work titled **`EVALUATION OF THE LEVEL OF COAGULATION FACTORS V AND VIII ON STORING FRESH FROZEN PLASMA AT DIFFERENT TEMPERATURES - A STUDY AT REGIONAL BLOOD BANK AND CEmONC CENTRE'** of the candidate **Dr.B.SAKTHIPRIYA** with registration Number 201631002 is for the award of **M.D (IH &BT)** in the branch of **M.D BRANCH – XXI (Immunohaematology & Blood Transfusion).** I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **2 percentage** of plagiarism in the dissertation.

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SAMPLE -70	BASELINE		0 HOURS		24 HOURS		72 HOURS		120 HOURS			BASELINE		0 HOURS		24 HOURS		72 HOURS		120 HOURS	
	PT(sec)	APTT	PT	APTT	PT	APTT	PT	APTT	PT	APTT	SAMPLE -30	PT	APTT	PT	APTT	PT	APTT	PT	APTT	PT	APTT
1	12.6	28.6	12.9	28.8	13.1	30	14	31.8	15	32.9	1	12.6	28.6	13.1	28.9	13.5	30	14.2	32	15	33.1
2	12.3	27.5	12.6	27.7	13.1	28.6	13.5	30.6	14.7	31.7	2	12.3	27.5	12.8	27.9	13.3	29.1	13.8	30.9	14.9	32
3	13.1	28.4	13.4	28.6	13.9	29.7	14.6	31.5	15.3	32.5	3	13.1	28.4	13.5	28.9	14	30.2	14.7	32	15.7	33.2
4	12.8	27.3	13.1	27.5	13.5	28.5	14.3	30.7	15	31.9	4	12.8	27.3	13.4	27.8	13.9	29.9	14.5	32.1	15.2	32.9
5	12.3	29.6	12.5	29.8	12.9	31	13.7	32.7	16.2	33.7	5	12.3	29.6	12.7	30	13.4	31.3	13.9	33	14.5	34
6	13.8	28.5	14.2	28.9	14.7	29.7	15.4	31.9	16.3	33	6	13.8	28.5	14.5	29.2	15	30.4	15.6	32.2	16.3	33.4
7	13.5	27.9	13.8	28.2	14.2	29.1	14.9	31.1	15.8	32.1	7	13.5	27.9	13.9	28.5	14.4	29.7	15	31.7	16	32.6
8	14.9	28.4	15.1	29.2	15.6	30.3	16.3	32.3	17	33.4	8	14.9	28.4	15.4	29.4	15.9	30.7	16.6	32.9	17.2	34.1
9	13.2	27.3	13.5	27.6	13.9	28.7	14.7	30.6	15.6	31.7	9	13.2	27.3	13.9	27.8	14.3	29	15.1	31.1	16	32.3
10	12.4	28.2	12.7	28.4	13.1	29.3	13.8	31.7	14.7	32.9	10	12.4	28.2	13	28.6	13.3	29.8	14.1	32	14.9	33.1
11	14.9	29.2	15.3	29.4	15.8	29.9	16.5	32.5	17.4	33.6	11	14.9	29.2	15.5	29.6	15.9	30.8	16.4	32.6	17.6	33.7
12	12.2	29.2	12.5	29.4	12.9	30.6	13.6	32.7	14.7	33.7	12	12.2	29.2	12.8	29.7	13.4	30.8	13.6	32.9	14.7	34
13	13.2	28.4	13.4	28.8	13.8	29.6	14.6	32	15.6	33.1	13	13.2	28.4	13.8	29	13.9	30.2	14.9	32.3	15.7	33.5
14	13.8	27.5	14.1	27.8	14.6	28.8	15.2	30.9	16.2	32	14	13.8	27.5	14.4	28.1	14.8	29.4	15.6	31.6	16.4	32.5
15	13.8	29.5	14.2	29.7	14.7	30.8	15.4	32.8	16.2	34	15	13.8	29.5	14.6	29.9	15.2	31	15.6	33.1	16.8	34.2
16	14.2	28.7	14.4	29.4	14.9	30.5	15.6	32.4	16.5	33.6	16	14.2	28.7	14.9	29.6	15.4	30.8	15.8	33	17	34.2
17	14.8	29.2	15.1	29.6	15.5	30.5	16.3	32.7	17.2	33.8	17	14.8	29.2	15.3	29.9	15.9	31	16.3	33.1	17	34
18	12.3	29.2	12.5	29.6	12.9	30.6	13.7	32.5	14.6	33.6	18	12.3	29.2	12.8	29.9	13.4	31.2	13.7	33.1	14.8	33.9
19	12.6	28.5	12.9	28.9	13.4	30	14	31.6	15.2	32.7	19	12.6	28.5	13.1	29.1	13.7	30.4	14.2	32.5	15.2	33.4
20	13.2	30.8	13.4	31	13.3	32.1	14.5	34	15.4	35.1	20	13.2	30.8	13.6	31.1	14	32.3	14.7	34.3	15.7	35.4
21	12.4	29.6	12.6	29.9	13.1	31	13.8	32.9	14.7	33.9	21	12.4	29.6	12.8	30	13.3	31.2	13.6	33.1	14.9	34
22	14.6	28.4	14.9	28.8	15.4	29.7	15.9	31.9	17	33	22	14.6	28.4	15.1	29.1	15.8	30.4	16	32.8	17	33.9
23	12.2	29.3	12.5	29.7	12.9	30.9	13.6	32.6	14.5	33.7	23	12.2	29.3	12.6	29.9	13	31	13.7	33.2	14.7	34.1
24	14.3	28.6	14.6	28.8	15.1	29.5	15.7	31.9	16.8	33.1	24	14.3	28.6	14.8	29.1	15.3	30.3	15.6	32.7	16.8	33.7
25	12.1	30.2	12.4	30.5	12.8	31.6	13.5	33.6	14.4	34.7	25	12.1	30.2	12.6	30.8	13.2	31.7	13.6	33.4	14	34.6
26	14.9	29.2	15.3	29.8	15.8	30.9	16.6	32.9	17.5	34	26	14.9	29.2	15.5	30	16	31.3	16.7	33	17.1	34.2
27	12.6	29.3	12.9	29.7	13.3	30.8	14.2	32.7	15.1	33.8	27	12.6	29.3	13	30	13.5	31.2	14.2	33.4	15	34.3
28	14.7	29.1	14.9	29.7	15.4	31	16.1	32.9	16.9	34	28	14.7	29.1	15.1	30.1	15.5	31.5	16	33.4	17.2	34.4
29	14.4	28.3	14.7	28.9	15.1	30	15.8	31.9	16.7	33.1	29	14.4	28.3	14.9	29.1	15.2	30.4	15.8	32.6	16.8	33.7
30	12.4	29	12.6	29.4	13.1	30.2	13.9	32.5	14.8	33.7	30	12.4	29	12.8	29.6	13.3	30.7	13.9	32.8	14.9	33.9
31	14.5	34.6	14.8	35	15.4	35.9	15.9	37.9	17	39	31	14.5	34.6	14.9	35.1	15.4	36.6	15.8	38.3	17	39.6
32	14.8	28.5	15.1	29.1	15.5	30	16.1	32.2	16.9	33.2	32	14.8	28.5	15.2	29.2	15.8	30.3	16	32.6	17.1	33.7
33	13.1	29.4	13.4	30	13.8	31.1	14.5	32.9	15.6	33.8	33	13.1	29.4	13.6	30.2	14.2	31.4	14.8	33.6	15.6	34.5
34	14	28.2	14.3	28.9	14.7	30	15.4	32	16.2	33.1	34	14	28.2	14.5	29.1	15	30.5	15.7	32.8	16.4	33.6
35	13.3	29.6	13.6	29.9	14.1	30.8	14.8	32.9	15.7	33.9	35	13.3	29.6	13.8	30.1	14.1	31.5	15	33.4	15.9	34.4
36	13.8	31.4	14.1	31.5	14.8	32.6	15.1	34.3	16	35.4	36	13.8	31.4	14.3	31.7	14.7	32.9	15.1	35	16.4	36
37	14.1	29.2	14.3	29.8	14.9	30.7	15.4	32.9	16.2	34	37	14.1	29.2	14.5	29.9	15.1	31	15.5	32.7	16.4	33.6
38	14.7	30.6	14.9	30.9	15.3	32	16.1	34	17.1	35.1	38	14.7	30.6	15.1	31	15.5	32.3	16.4	34.1	17	35.3
39	13.5	30.2	13.8	30.8	14.2	31.7	14.9	33.9	15.8	35	39	13.5	30.2	14	31	14.7	32.3	15.1	33.9	15.8	35.3
40	12.6	31.3	12.9	31.8	13.4	32.8	14.2	34.7	15.1	35.8	40	12.6	31.3	13.1	32.1	13.5	33.3	14.2	35.7	15.3	36.6

SAMPLE -70^{0}C	BLOOD GROUP	BASELINE			0 HOUR			24 HOURS			72 HOURS			120 HOURS		
		FIB mg/dl	F V IU/ml	F VIII IU/ml	FIB mg/dl	F V IU/ml	F VIII IU/ml	FIB mg/dl	F V IU/ml	F VIII IU/ml	FIB mg/ml	F V IU/ml	F VIII IU/ml	FIB mg/dl	F V IU/ml	F VIII IU/ml
1	B POS	296.4	1.02	0.96	294.3	0.97	0.94	290.2	0.92	0.87	280.6	0.84	0.76	274.2	0.79	0.69
2	B POS	303.2	0.94	1.05	301.7	0.94	0.96	297.2	0.88	0.9	291.5	0.82	0.79	286.4	0.75	0.72
3	A1 POS	284.2	1.04	0.89	282.3	0.97	0.86	277.6	0.94	0.82	274.5	0.85	0.73	270.2	0.74	0.64
4	B POS	276.8	1.14	0.96	273.8	1.02	0.92	269.2	0.96	0.84	265.9	0.89	0.72	257.4	0.77	0.62
5	B POS	292.2	1.32	1.17	288.3	1.22	1.02	284.2	1.09	0.96	276.8	0.96	0.87	270.8	0.88	0.74
6	B POS	284.8	1.01	0.97	281.2	0.97	0.93	277.2	0.91	0.86	269.6	0.84	0.79	262.5	0.76	0.68
7	B POS	278.2	0.96	0.87	273.1	0.94	0.84	268.5	0.87	0.78	263.7	0.82	0.69	260.7	0.76	0.61
8	B POS	293.2	0.98	1.06	288.5	0.96	0.98	284.7	0.89	0.88	279.6	0.81	0.79	272.8	0.74	0.67
9	B NEG	282.6	1.32	0.98	278.4	1.24	0.94	274.8	1.12	0.86	270.4	0.96	0.74	265.4	0.87	0.63
10	B NEG	268.4	0.94	0.89	265.2	0.91	0.84	262.8	0.87	0.79	258.9	0.82	0.71	252.8	0.74	0.65
11	A1 POS	285.8	0.96	0.84	282.4	0.94	0.79	278.4	0.87	0.71	273.6	0.82	0.63	267.8	0.77	0.56
12	A1 POS	291.6	0.92	1.03	286.4	0.88	0.95	280.6	0.82	0.87	275.2	0.76	0.79	272.8	0.71	0.68
13	B POS	288.4	1.02	0.96	284.3	0.97	0.89	279.8	0.91	0.81	272.3	0.83	0.74	267.9	0.76	0.65
14	A POS	302.6	1.16	1.04	296.8	1.08	0.97	290.7	1.02	0.89	284.8	0.96	0.81	279.8	0.89	0.72
15	A POS	292.2	1.04	0.98	287.5	0.98	0.91	283.5	0.92	0.83	279.4	0.88	0.74	275.5	0.81	0.63
16	A POS	279.8	0.98	0.86	274.8	0.93	0.79	270.2	0.87	0.71	264.8	0.81	0.63	259.3	0.74	0.54
17	A POS	274.2	0.95	0.83	270.3	0.91	0.75	267.4	0.86	0.68	264.3	0.79	0.61	258.6	0.72	0.52
18	A POS	288.4	0.89	0.96	283.2	0.83	0.89	279.2	0.77	0.81	275.8	0.71	0.73	270.4	0.65	0.63
19	O POS	272.9	0.84	0.86	268.4	0.81	0.79	261.7	0.74	0.71	256.9	0.68	0.63	251.7	0.62	0.54
20	A POS	296.4	0.93	0.88	290.4	0.89	0.82	286.2	0.81	0.74	281.7	0.76	0.69	276.8	0.71	0.61
21	A POS	278.4	0.88	0.94	274.3	0.83	0.87	270.2	0.78	0.79	266.7	0.72	0.71	262.3	0.67	0.62
22	A POS	296.2	0.97	0.86	290.3	0.92	0.79	286.9	0.88	0.72	281.4	0.81	0.63	277.2	0.74	0.56
23	A POS	304.2	1.08	0.98	298.4	1.04	0.92	294.3	0.98	0.84	289.7	0.92	0.76	283.4	0.86	0.68
24	B POS	282.6	0.89	0.92	278.3	0.84	0.87	274.5	0.79	0.81	270.6	0.74	0.72	264.7	0.67	0.61
25	B POS	297.4	1.02	0.97	292.7	0.98	0.93	287.8	0.93	0.85	283.4	0.87	0.76	278.9	0.81	0.64
26	B POS	287.6	0.86	0.88	284.8	0.82	0.84	280.6	0.77	0.78	276.7	0.72	0.69	272.3	0.64	0.61
27	B POS	306.2	1.12	1.02	304.7	1.08	0.96	298.9	1.02	0.89	294.5	0.94	0.79	289.7	0.89	0.68
28	B POS	288.2	1.06	0.94	285.2	1.02	0.89	282.4	0.97	0.81	279.6	0.92	0.73	275.5	0.86	0.65
29	O POS	278.6	0.83	0.91	276.2	0.79	0.87	272.3	0.74	0.79	267.8	0.68	0.71	262.7	0.63	0.61
30	O POS	285.4	0.91	0.89	282.5	0.87	0.84	278.6	0.83	0.76	273.7	0.78	0.68	268.8	0.72	0.59
31	A1 POS	272.6	0.94	0.87	268.2	0.91	0.82	262.7	0.87	0.74	257.9	0.82	0.63	252.3	0.77	0.54
32	B POS	294.2	0.97	0.94	288.4	0.93	0.88	283.5	0.89	0.81	279.8	0.84	0.73	274.5	0.78	0.65
33	O POS	291.3	0.88	0.84	287.2	0.83	0.79	284.5	0.78	0.71	280.2	0.72	0.63	276.8	0.65	0.52
34	O POS	284.9	0.92	0.87	280.5	0.87	0.81	276.9	0.83	0.74	272.7	0.78	0.67	268.2	0.71	0.59
35	O POS	287.6	0.89	0.82	283.4	0.84	0.76	279.4	0.79	0.69	275.4	0.73	0.61	271.2	0.67	0.51
36	B POS	314.8	1.23	0.91	308.4	1.16	0.87	304.7	1.06	0.79	297.9	0.98	0.71	293.2	0.91	0.62
37	B POS	294.6	1.02	0.88	289.2	0.98	0.82	285.7	0.94	0.74	282.7	0.87	0.63	278.5	0.81	0.54
38	B POS	304.2	0.96	0.92	298.7	0.92	0.87	294.5	0.88	0.8	289.6	0.81	0.72	284.7	0.75	0.61
39	B POS	278.5	0.88	0.96	274.2	0.83	0.89	270.2	0.79	0.82	266.5	0.73	0.74	262.4	0.68	0.65
40	B POS	306.7	1.16	1.03	296.7	1.08	0.97	291.2	1.02	0.89	287.3	0.96	0.81	282.3	0.89	0.72

SAMPLE -30^{0}C	BLOOD	BASELINE			0 HOUR			24 HOURS			72 HOURS			120 HOURS		
	GROUP	FIB mg/dl	F V IU/ml	F VIII IU/ml	FIB mg/dl	F V IU/ml	F VIII IU/ml	FIB mg/dl	F V IU/ml	F VIII IU/ml	FIB mg/ml	F V IU/ml	F VIII IU/ml	FIB mg/dl	F V IU/ml	F VIII IU/ml
1	B POS	296.4	1.02	0.96	292.7	0.96	0.93	288.6	0.91	0.85	279.4	0.82	0.73	270.3	0.74	0.63
2	B POS	303.2	0.94	1.05	298.5	0.92	0.94	294.6	0.87	0.88	289.6	0.79	0.78	282.3	0.72	0.67
3	A1 POS	284.2	1.04	0.89	281.7	0.95	0.85	276.8	0.91	0.79	273.4	0.84	0.71	269.7	0.73	0.63
4	B POS	276.8	1.14	0.96	272.5	0.97	0.88	268.4	0.92	0.81	263.7	0.86	0.69	255.2	0.74	0.58
5	B POS	292.2	1.32	1.17	287.6	1.15	0.99	283.5	1.08	0.94	275.8	0.93	0.81	268.7	0.84	0.69
6	B POS	284.8	1.01	0.97	280.2	0.96	0.93	276.2	0.89	0.87	264.8	0.81	0.78	257.3	0.73	0.67
7	B POS	278.2	0.96	0.87	271.6	0.91	0.81	266.5	0.84	0.75	260.2	0.78	0.64	254.8	0.69	0.56
8	B POS	293.2	0.98	1.06	286.9	0.94	0.96	282.5	0.86	0.85	276.3	0.79	0.74	269.2	0.71	0.64
9	B NEG	282.6	1.32	0.98	276.9	1.16	0.91	273.2	1.08	0.81	267.4	0.91	0.72	258.9	0.76	0.63
10	B NEG	268.4	0.94	0.89	264.3	0.89	0.82	260.7	0.82	0.74	255.2	0.76	0.65	246.7	0.69	0.54
11	A1 POS	285.8	0.96	0.84	280.7	0.91	0.77	275.6	0.84	0.69	270.8	0.74	0.61	263.9	0.65	0.52
12	A1 POS	291.6	0.92	1.03	285.8	0.86	0.93	277.8	0.81	0.84	272.4	0.72	0.75	263.8	0.67	0.63
13	B POS	288.4	1.02	0.96	277.6	0.94	0.87	273.5	0.86	0.78	268.3	0.79	0.69	261.2	0.71	0.61
14	A POS	302.6	1.16	1.04	294.8	1.02	0.94	289.4	0.94	0.85	283.2	0.86	0.76	277.2	0.76	0.64
15	A POS	292.2	1.04	0.98	285.6	0.95	0.89	280.7	0.87	0.81	273.2	0.81	0.72	267.5	0.74	0.61
16	A POS	279.8	0.98	0.86	272.5	0.91	0.76	266.3	0.85	0.67	258.7	0.78	0.59	252.7	0.71	0.51
17	A POS	274.2	0.95	0.83	268.7	0.87	0.74	262.7	0.81	0.65	257.4	0.72	0.59	248.2	0.63	0.51
18	A POS	288.4	0.89	0.96	281.5	0.81	0.87	276.5	0.74	0.78	269.8	0.68	0.63	263.8	0.61	0.53
19	O POS	272.9	0.84	0.86	267.2	0.79	0.76	260.2	0.72	0.69	255.4	0.64	0.61	250.2	0.57	0.51
20	A POS	296.4	0.93	0.88	289.2	0.87	0.79	284.7	0.8	0.71	278.7	0.74	0.63	270.4	0.67	0.54
21	A POS	278.4	0.88	0.94	272.8	0.82	0.84	267.5	0.76	0.72	260.4	0.69	0.67	254.3	0.61	0.58
22	A POS	296.2	0.97	0.86	289.4	0.91	0.77	283.4	0.84	0.68	277.4	0.76	0.61	270.5	0.68	0.51
23	A POS	304.2	1.08	0.98	296.5	1.02	0.89	287.5	0.93	0.81	281.7	0.85	0.72	276.4	0.76	0.61
24	B POS	282.6	0.89	0.92	277.6	0.82	0.83	272.3	0.78	0.72	267.4	0.71	0.63	261.2	0.64	0.54
25	B POS	297.4	1.02	0.97	290.5	0.94	0.88	284.3	0.88	0.79	279.4	0.81	0.72	272.3	0.74	0.61
26	B POS	287.6	0.86	0.88	282.5	0.81	0.79	277.6	0.75	0.71	271.8	0.69	0.63	262.8	0.62	0.54
27	B POS	306.2	1.12	1.02	302.6	0.98	0.94	296.7	0.91	0.86	290.4	0.86	0.78	283.4	0.79	0.65
28	B POS	288.2	1.06	0.94	284.7	0.98	0.87	279.8	0.93	0.79	274.6	0.86	0.72	268.7	0.78	0.63
29	O POS	278.6	0.83	0.91	272.6	0.76	0.84	268.4	0.69	0.76	261.4	0.63	0.65	254.8	0.57	0.54
30	O POS	285.4	0.91	0.89	281.7	0.86	0.82	274.2	0.81	0.72	265.9	0.76	0.65	259.3	0.69	0.56
31	A1 POS	272.6	0.94	0.87	267.4	0.87	0.79	261.2	0.79	0.71	254.6	0.72	0.61	248.7	0.63	0.54
32	B POS	294.2	0.97	0.94	287.8	0.89	0.86	278.8	0.84	0.78	267.8	0.76	0.71	259.3	0.65	0.63
33	O POS	291.3	0.88	0.84	286.5	0.84	0.76	282.3	0.77	0.69	276.7	0.71	0.61	269.4	0.67	0.52
34	O POS	284.9	0.92	0.87	279.6	0.85	0.79	273.4	0.81	0.72	268.3	0.75	0.64	262.7	0.69	0.56
35	O POS	287.6	0.89	0.82	282.4	0.83	0.74	276.8	0.79	0.67	269.3	0.71	0.61	262.4	0.63	0.54
36	B POS	314.8	1.23	0.91	306.4	1.08	0.84	298.4	0.98	0.76	292.5	0.89	0.67	281.9	0.72	0.59
37	B POS	294.6	1.02	0.88	287.5	0.96	0.79	282.5	0.89	0.71	276.2	0.81	0.62	270.4	0.74	0.54
38	B POS	304.2	0.96	0.92	296.8	0.89	0.84	288.7	0.82	0.72	280.4	0.75	0.64	273.6	0.68	0.52
39	B POS	278.5	0.88	0.96	272.8	0.81	0.87	268.5	0.75	0.78	261.8	0.67	0.69	254.7	0.61	0.59
40	B POS	306.7	1.16	1.03	294.2	1.04	0.94	288.7	0.96	0.84	280.5	0.89	0.76	274.5	0.82	0.64